IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Applicants: | C. Lambeth et al. | Attorney Docket No.: | WEYE115226 |
| Application No.: | 09/618,307 | Art Unit: 1638 / Confirmation No: | 9512 |
| Filed: | July 18, 2000 | Examiner: David T. Fox | |
| Title: | POLLEN POLYMIX PLANT BREEDING METHOD UTILIZING MOLECULAR PEDIGREE ANALYSIS | | |

# DECLARATION OF CLEMENTS C. LAMBETH

Seattle, Washington 98101

August 3, 2006

TO THE COMMISSIONER FOR PATENTS:

I, Dr. Clements C. Lambeth, declare as follows:

1. I am a co-inventor of the subject matter in the above-identified patent application and I am employed by Weyerhaeuser Company as a Tree Improvement Department Manager and Senior Scientific Advisor.

2. A copy of my curriculum vitae is appended hereto as Attachment B.

3. I have considered the Office Action dated October 18, 2004 and the decision by the Board of Patent Appeals and Interferences, dated June 6, 2006, issued in the above-identified application. It is my understanding that the Board has reversed the Examiner's rejections of claims in the application based on lack of written description and lack of enablement, and has affirmed the rejection of claims based on obviousness in view of Bridgwater (1992) in "Handbook of Quantitative Forest Genetics," Kluwer Academic Pub., Dordrect, The Netherlands, pp. 69-95, in view of El-Kassaby and Ritland (1992) *Theor. Appl. Genet. 83*(6-7):752-8 and Stoehr et al. (1998) *Can. J. For. Res. 28*:187-95.

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4. It is well known by those with knowledge in the field of tree breeding that it takes a very long time to validate a breeding method with tree species due to their long life span and the long time it takes to induce flowers. Therefore, in order to validate some of the benefits of the tree breeding method claimed in our pending patent application serial number 09/618,307, referred to as polymix breeding with parental analysis (PMX/WPA), my colleagues and I, with the aid of a contract laboratory Select Breed Services, designed and conducted a quantitative genetics simulation study in 2002 comparing PMX/WPA to a conventional full-sib breeding system. The simulation study, described in the research report attached hereto as Attachment C, assumed certain population statistics, heritability, inbreeding effects and selection through eleven generations. The simulation study demonstrated that PMX/WPA resulted in greater genetic gain over ten generations of recurrent selection under both restricted and unrestricted inbreeding scenarios than full-sib breedings, at a significantly reduced cost. In addition, the parental breeding values were more accurately estimated under the PMX/WPA system, implying that orchard roguing and family allocations based on progeny performance would be more accurate than with the conventional full-sib systems.

5. My colleagues and I conducted a study in 2005 in order to test the contribution of males in a polymix breeding system with parental analysis (PMX/WPA). In this study, ten females were pollinated with a 35-male pollen mix and ~ 44 progeny per female were subjected to paternity analysis. Paternity assignment in this study was based on the use of 12 chloroplast and 3 nuclear microsatellite markers, which allowed for the unambiguous identification of 70% of the fathers and approximately 80% of the possible 350 crosses had at least a partial paternity assignment. The results of this study are presented in FIGURE 1, (appended hereto) and demonstrated that males contributed to fertilization in a near random fashion and the frequency distribution of the contribution of males to the progeny was excellent. Because of this random



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distribution of paternal success, many effective crosses were created, with nearly 80% of the possible 350 crosses having at least a partial paternity assignment. In contrast, in a circular, full-sib mating system, a preferred method in the field, only 70 crosses would be created. The random distribution of paternal success demonstrated in this study resulted in more crosses than would be possible in a traditional breeding system, which would therefore produce more diversity than traditional breeding systems. The increase in diversity that is made possible with the PMX/WPA breeding method allows for more choices of unrelated or weakly related trees for inclusion in a breeding group for use in a next generation of tree breeding to allow for increased genetic gain through selection and better inbreeding control.

The results of this study also demonstrated that crosses between closely related pollens resulted in a low frequency, thereby adding an additional advantage to the PMX/WPA method. In this study, some of the pollen in the polymix was related to the female (self, full-sib, parent). Based on a mathematical calculation assuming equal contribution of all males, the expectation in this study was 1.25 progeny per each of the 350 potential crosses. However, what was actually observed was that unrelated crosses averaged 1.36 progeny per cross while self and close relative (full-sibs, parent-offspring) crosses had only 0.37 and 0.61 progeny per cross. Therefore the results in this study showed that closely related pollens were at a fertilization disadvantage and/or their progeny were selected against in the early stages of life. It is well known by those with knowledge in tree breeding that mating of close relatives is not desirable in loblolly pine due to inbreeding depression in this highly out-crossed species. Therefore, the low frequency of inbred progeny observed in this study demonstrates an additional advantage of practicing the claimed method which further increases the potential for genetic gain that can be achieved using the claimed PMX/WPA tree breeding method.



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6. The PMX/WPA tree breeding method satisfies a long-felt need in the field of tree breeding. Prior to the publication of our journal article describing the claimed PMX/WPA breeding method (Lambeth, C.C. et al., *Theor. Appl. Genet. 103*:930-943 (2001)), there was a long standing need for a workable solution to the use of a polymix breeding scheme in isolation from other crossing schemes in tree breeding programs. The potential value of polymix breeding method has been long recognized by authors in the field of tree breeding. (See for example, Shelbourne C.J.A., "Tree Breeding Methods," Tech. Pap. No. 55, New Zealand Forest Service, (1969), attached hereto as Attachment D; and Burdon R.D. and Shelbourne C.J.A., "Breeding populations for Recurrent Selection: Conflicts and Possible Solutions," *NZ J. For. Sci. 1*(2): 174-193 (1971), attached hereto as Attachment E). However, the limitations of polymix breeding have also long been recognized and include lack of full pedigree control, which limits genetic gain due to lack of breeding value on the male parent, and inability to control inbreeding levels. These limitations have led to the predominant view in the field of tree breeding (prior to the publication of the methods of the instant invention) that polymix breeding should only be used for estimation of breeding value of the parent and not as a basis for selection of the next generation of breeding (see for example, McKeand, S.E. and Bridgwater, F.E., "Third Generation Breeding Strategy for the North Carolina State University-Industry Cooperative Tree Improvement Program," In Proc. IUFRO Resolving Tropical Forest Resource Concerns Through Tree Improvement, Gene Conservation and Domestication of New Species, Cali, Columbia pp. 234-240 (1992), attached hereto as Attachment F; Burdon, R.D. and van Buijtenen, J.P., "Expected Efficiencies of Mating Designs for Reselection of Parents," *Can. J. For. Res. 20*:1664-1671 (1990), attached hereto as Attachment G).

For example, as described in McKeand and Bridgwater, (1992) (see Attachment F), university based cooperative tree breeding programs in the United States have used a dual



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complementary breeding system in which polymix crossing is used to estimate breeding values and full-sib crossing is done to produce a population for selecting the next generation of trees. This dual complementary breeding system is known to be costly and time consuming. However, prior to the publication in 2001 (Lambeth et al., 2001) of the method of the claimed invention, there was no proposed solution to combine the polymix breeding system with pedigree analysis to produce the method as claimed in the present invention.

7. The PMX/WPA breeding method as claimed in the pending claims 20-31 of application serial number 09/618,307 solves the limitations of the polymix breeding system and eliminates the need for the dual complementary breeding system described above in which polymix crossing is used to estimate breeding values and full-sib crossing is done to produce a population for selecting the next generation of trees. The PMX/WPA breeding method is also less expensive and has the potential to provide more genetic gain in a shorter time span than the dual complementary breeding system.

In response to our publication in 2001 (Lambeth et al., 2001) describing the method of the claimed invention, there has been acceptance of our breeding method by those in the field of tree breeding as evidenced by favorable comments we have received from scientists in the field, requests for collaborations, and copying of our breeding methods by others, as described in more detail below.

The reaction my colleagues and I have received from several scientists in the field is that the claimed invention is a very creative solution to the problem of tree breeding and one in which many of them are interested in due to one or more advantages of simplicity, speed, excellent estimates of both parental and individual progeny breeding values, greater opportunity to reduce inbreeding and greater genetic gain than conventional full-sib systems. The following



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representative examples are provided as evidence of acceptance by scientists in the field of tree breeding, collaboration and/or copying of our PMX/WPA breeding method by others:

- Dr Dudley Huber, Quantitative Geneticist and Professor at the University of Florida has described our method as a novel and effective approach to an age-old problem (personal communications).
- Dr. Satish Kumar and his New Zealand and Australian colleagues at ENSIS are very interested in our method and mention our publication as a "leading paper" in an email communication dated July 20, 2006, attached hereto as Attachment H.
- Dr Terry Stranger, geneticist with SAPPI Timber Company in South Africa and his colleagues from the University of Pretoria have discussed the PMX/WPA breeding method with me and have launched a research effort to test the efficacy of our breeding method in commercial eucalypt species, as described in a poster article by Minique H. et Castro et al., 2004, attached hereto as Attachment I.
- Boise Cascade Corporation has contracted with co-inventor Nicholas Wheeler to conduct a study on the utility of our PMX/WPA method in popular breeding and a paper was published on the results, Wheeler N. et al., *Tree Genomics and Genomes 2*:53-60 (2006), attached hereto as Attachment J.
- Dr. Dario Grattapaglia has cited our paper describing the PMX/WPA method (Lambeth et al. 2001), and has employed a similar strategy for the improvement of eucalypt seed orchards in Brazil (see Grattapaglia et al., *Theor. Appl. Genet. 109*:192-199 (2004), attached hereto as Attachment K.
- Dr. Rowland Burdon, Senior Research Scientist at the Forestry Research Institute in New Zealand, has cited our PMX/WPA breeding method and mentions its value in the control of inbreeding in Burdon et al., IUFRO Division 2



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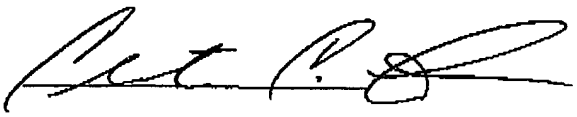
Proceedings, "Forest Genetics and Tree Breeding in the Age of Genomics: Progress and Future," Charleston S.C. November 1-5, 2004, pages 75-85, attached hereto as Attachment L.

8. All statements made herein and of my own knowledge are true, and all statements made on information and belief are believed to be true; and further, these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Respectfully submitted,



Clements C. Lambeth, Ph.D.

Date: 8-3-06

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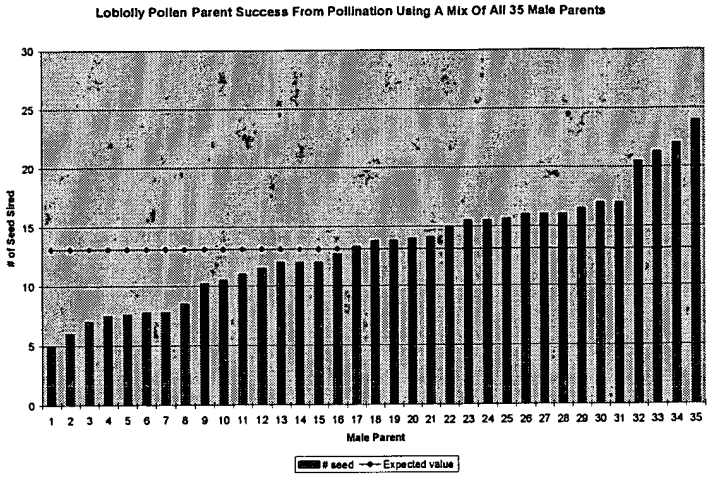
FIGURE 1

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# Clements C. Lambeth, Senior Scientific Advisor

## *CAREER OBJECTIVE*

To work in a goal-driven, forestry research organization that places strong emphasis on technology implementation, environmental stewardship and employee job satisfaction.

## *EDUCATION AND TRAINING*

**Degrees Earned**

- ⇒ 1972 - B.S. in Forestry, Stephen F. Austin University, Nacogdoches, TX (Outstanding Senior Award, Texas Forest Service Scholarship Recipient)
- ⇒ 1974 - M.S. in Forest Genetics, Yale University, New Haven, CT (Research Fellow Recipient)
- ⇒ 1979 - Ph.D. in Forest Genetics, N.C. State University, Raleigh, NC (Weyerhaeuser Graduate Studies Program Recipient)

**Other Training**

- ⇒ Management Skills
- ⇒ Podium Power (Public Speaking)
- ⇒ Total Quality Modules (Weyerhaeuser)
- ⇒ Managing for Continuous Improvement (Tennessee Associates)
- ⇒ Covey's 7 Habits of Successful People
- ⇒ People Skills
- ⇒ Advanced SAS (Statistical Analysis System)
- ⇒ Biotechnology in Forestry Short Course – University of Florida
- ⇒ Various MicroSoft Products Including MicroSoft Project, Excel, Word, PowerPoint, Access
- ⇒ Spanish proficiency through intensive courses and on the job usage
- ⇒ Managing Multiple Projects, Objectives and Deadlines
- ⇒ Diversity Training
- ⇒ Self-Directed Work Teams
- ⇒ Safety Including Behavior, CPR, First-Aid, etc.

**Other Interests** Travel, Canoeing, Reading, Coffee Roasting, Building

# *EXPERIENCE HIGHLIGHTS*

**Weyerhaeuser** Hot Springs, Arkansas

**1992-Present** **Tree Improvement Department Manager & Senior Scientific Advisor**

**Roles and Responsibilities (Managed up to 14 scientists and technicians):**

- ⇒ Lead a team that develops genetically improved stock for 4.5 million acres in the southeastern U.S.
- ⇒ Provide periodic presentations and reviews to Senior Timberlands leadership for decision-making regarding the development, production and deployment of genetically improved stock in the southeastern U.S.
- ⇒ Lead the somatic embryogenesis clonal development and testing program
- ⇒ Provide the needed information to Southern Timberlands area foresters for successful deployment of genetically improved stock
- ⇒ Represent Weyerhaeuser on executive committees of 4 University-based tree improvement and research cooperatives
- ⇒ Provide consultation to Uruguay and Saskatchewan timberlands leaders on tree improvement strategies and help develop long-term plans
- ⇒ Conduct research to improve the efficiency and genetic gain in breeding, testing, selection and orchard processes
- ⇒ Serve on a company-wide Clonal Forestry Implementation team that develops the strategy for implementation of cloning on all Weyerhaeuser land
- ⇒ Serve on a Seed and Orchard Functional Team that studies alternatives and makes recommendations on changes in strategy and operational practices that improve growth, cost and/or quality of Weyerhaeuser's Southern Timberlands through tree improvement and seed orchards

**Key Accomplishments and Communications:**

- ⇒ Presented a Southern Tree Improvement Strategy that was approved by the Senior VP of Timberlands
- ⇒ Wrote the Southern Tree Improvement Plan which details strategy as well as breeding, testing and selection activities and their timelines for Weyerhaeuser's 4 million acres in 9 states
- ⇒ Led in the integration of Proctor and Gamble, MacMillan Bloedel, Cavenham and Willamette tree improvement programs

- ⇒ Played a key role in the development of a widely used Landform Table which details the cost and benefit of various silvicultural and tree improvement options for all southeastern landforms and allows the user to test the economic benefits of single or multiple early rotation, stand management options
- ⇒ Developed an electronic south-wide seed catalog that is used by Operations and Research personnel as a planning tool for family deployment, new orchards and breeding work
- ⇒ Played a key role in development of a long-term orchard production strategy that was approved by the Southern Timberlands VP
- ⇒ Led a team review with the Southern Timberlands VP and the lead team which prompted Southern Timberlands to make a potentially $50 million decision to plant North Carolina stock in Ark/Okla & NLouis.
- ⇒ Developed a rooted cuttings research and implementation strategy that was approved by the Timberlands Senior VP and Southern Timberlands VP
- ⇒ Managed a team that developed improved outdoor rooting techniques and mechanization of steps in rooted cuttings
- ⇒ Developed a new breeding concept (PMX/WPA) to increase genetic gain and efficiency in improvement programs using paternity analysis
- ⇒ Led in the implementation of top grafting, a new technology for breeding, new orchards and crown replacement in operational orchards
- ⇒ Led in the implementation of control mass pollination (CMP) in operational orchards including a strategy presentation that was approved by the Southern Timberlands VP
- ⇒ Led a multi-department (forestry, analytical sciences, solidwood, pulp and paper) team effort in a study of the inheritance and gain potential of wood quality traits in loblolly pine that led to incorporating them into the tree improvement program
- ⇒ Developed tree improvement strategies for Saskatchewan Timberlands and COLONVADE in Uruguay and made presentations to the forest management leaders in those areas
- ⇒ Worked with a team of outside scientists to redefine seed source transfer guidelines for southern pines

**1989-1992**

**Smurfit Group** Jacksonville, Florida
**International Forestry R&D Manager**

**Roles and Responsibilities (Functional responsibility reporting to Timberlands VP's in Colombia, Venezuela, Mexico and the U.S.):**

- ⇒ Coordinated a multi-disciplinary and multi-country research team aimed at improving operations and yield in Latin America and the U.S.
- ⇒ Advised the presidents and vice-presidents of Colombia, Venezuela

and Mexican Timberlands organizations on R&D direction and led projects needing international coordination

⇒ Arranged for outside consultants as needed when internal skills were lacking in developing forestry programs

⇒ Conducted tree improvement research for Container Corporation in Florida, Alabama and Georgia

**Key Accomplishments and Communications:**

⇒ Developed breeding strategies for Colombia and Venezuela for several hardwood and pine species

⇒ Conducted on-the-ground initiation of the *Gmelina* and *Eucalyptus* clonal programs in Venezuela

⇒ Conducted research in controlled mass pollination in seed orchards

⇒ Initiated the testing of exotic pine species in Florida

⇒ Coordinated the publication of the Smurfit Group Forestry Research Report series

⇒ Organized an international conference of the IUFRO Tropical Tree Breeding and Testing group in Cartagena and Cali, Colombia

**1985-1989**

**Smurfit Carton de Colombia/Container Corp.** Cali, Colombia
**Director of Forestry R&D**

**Roles and Responsibilities (Managed up to 10 scientists and technicians):**

⇒ Advised company leadership in Colombia and Venezuela on research and reforestation activities

⇒ Led a multi-disciplinary team researching forest nutrition, competition control, hybrid and species testing, vegetative propagation, and tree improvement of pine and *Eucalyptus* species

⇒ Benchmarked tropical pine and eucalypt research programs in Africa, South America and the Orient

⇒ Coordinated the classification of new species and description of species diversity in the Bajo Calima rainforest on the Pacific Coast

**Key Accomplishments and Communications:**

⇒ Developed a long-term cloning strategy, sold it to upper management and led a team implementation effort in a highly successful cloning program in *Eucalyptus* with commendation from the president

⇒ Conducted research and made recommendations that led to changes

in species and provenances with large gains in growth rate and value in company plantations

- ⇒ Launched the forest nutrition research program
- ⇒ Established tree improvement programs and new seed orchards for *Pinus kesiya, Pinus tecunumanii* and *Pinus Patula*
- ⇒ Began an advanced education program for Colombian foresters with management and research potential

**1974-1985**

**Weyerhaeuser** Hot Springs, Arkansas

**Tree Improvement R&D Project Leader (starting as a technician in Centralia, Washington)**

**Roles and Responsibilities:**

- ⇒ Led a team conducting research to improve breeding, testing, selection, and orchard processes
- ⇒ Led in the allocation of genetic stock for all Weyerhaeuser holdings in the southeastern U.S.
- ⇒ Helped set tree improvement strategies for the southeastern U.S.

**Key Accomplishments and Communications:**

- ⇒ Conducted groundbreaking research in early genetic testing including the development of a juvenile-mature correlation prediction model that has been used by several international researchers
- ⇒ Conducted research on plot design leading to significant changes in test design
- ⇒ Conducted research on accelerated breeding of small elite breeding populations that led to changes in breeding population structure and long term strategies
- ⇒ Conducted research that defined differences in growth and drought tolerance of local and nonlocal seed sources and led a team Seed Source Movement Review with the Senior VP of Timberlands leading to provenance changes and high NPV potential for 1.5 million acres in Arkansas/Oklahoma
- ⇒ Developed a unique and effective system for drought resistance screening of genetic stock

# *RESEARCH LEADERSHIP AND TECHNICAL EXPERTISE*

## Honors and Awards

- ⇒ Adjunct Professor (current), North Carolina State University
- ⇒ Adjunct Professor, Oklahoma State University, 1984-1986
- ⇒ Adjunct Professor, Mississippi State University, 1984-1986
- ⇒ Honor Societies: Alphi Chi, Xi Sigma Pi (Forestry), Gamma Sigma Delta (Agriculture)
- ⇒ CAMCORE Lifetime Honorary Advisory Board Member
- ⇒ CAMCORE Executive Committee Member 2000-2004
- ⇒ Invited speaker at 19 professional conferences
- ⇒ Weyerhaeuser Outstanding Achievement Award, 1982
- ⇒ Presidential Commendation For Clonal Forestry Program at Smurfit Carton de Colombia
- ⇒ Forest Science article "Juvenile-Mature Correlations In Pinaceae And Implications For Early Selection" cited over 150 times in other scientific articles
- ⇒ Nomination for and election to position of "Senior Scientific Advisor" - a competitive and peer-reviewed position

***Recent Weyerhaeuser Special Recognition Awards***

- ⇒ 1998 Weyerhaeuser Special Recognition Award – for contributions to the Southwide Early Rotation Functional Team
- ⇒ 1999 Weyerhaeuser Special Recognition Award – for leading the Seed Source Movement Review with positive results
- ⇒ 2000 Weyerhaeuser Special Recognition Award – for contributions to development of long term tree improvement strategies for Southern Regeneration
- ⇒ 2001 Weyerhaeuser Special Recognition Award – for contributions to integrating clonal testing and review of D-fir advanced generation strategies
- ⇒ 2002 Weyerhaeuser Special Recognition Award – for development of the electronic Seed Catalog which has facilitated key regeneration decisions for Southern Timberlands
- ⇒ 2003 Nominated for Scientist 7 (Senior Research Scientist), a competitive position
- ⇒ 2003 Weyerhaeuser Special Recognition Award – for contributions to

developing and presenting the results that led to business buyoff in developing high MOE genetic stock

**Other Professional Activities**

- ⇒ Served on graduate and undergraduate committees at 4 universities
- ⇒ Chairman, North American Quantitative Forest Genetics Group
- ⇒ CAMCORE Technical Committee member
- ⇒ Chair, IUFRO Section On Breeding and Testing Tropical Species – 1988 to 1993 (led the organization of the international conference in Colombia in 1992 with over 100 participants from around the world)
- ⇒ Short Course Instructor (in Spanish) – CATIE, Costa Rica – 1991
- ⇒ Short Course Instructor (in Spanish) – DIRENA , Nicaragua – 1992
- ⇒ Southern Forest Tree Improvement Committee – 1991 to 2001
- ⇒ Chair, IEG-40 (Forest Genetics) – 1995-1997
- ⇒ Review manuscripts for several scientific journals such as Forest Science, Forest Genetics, Theoretical and Applied Genetics, Southern Journal of Applied Forestry, Canadian Journal of Forestry Research, Silvae Genetica, Journal of Tropical Forestry
- ⇒ Book chapter reviews for two books by Dr. Bruce Zobel
- ⇒ Book chapter review (Forest Genetics) for Dr. Tim White
- ⇒ Occasional reviewer of Forest Service and University Research Programs

# *REFERENCES*

**External**

- ⇒ Dr. Bruce Zobel, Professor Emeritus, N.C. State University
- ⇒ Dr. Bob Kellison, Director, N.C. Forest Biotechnology Center
- ⇒ Dr. Bill Dvorak, Professor/CAMCORE Director, N.C. State University
- ⇒ Rafael Diaz, President, Smurfit Carton de Venezuela
- ⇒ Dr. Tim White, Professor/CFGRP Director, University of Florida

**Internal (Weyerhaeuser)**

- ⇒ Dr. Rex McCullough (retired), Vice-President Forestry R&D
- ⇒ Dr. Howard Duzan, Director, Southern Forestry R&D
- ⇒ Dr. Peter Farnum, Vice-President Forestry R&D
- ⇒ Dr. Christine Dean, Director, Western Forestry R&D

# *PUBLICATIONS*

## Recent Popular (Company) Articles

⇒ Generations of Quality: The Tree Improvement Story (Weyerhaeuser Timberlands Special Report)

⇒ Tree Improvement ... The Best Value For Your Money (Weyerhaeuser - Tree Growing Times)

⇒ Ideal Trees That Are Infinitely Reproducible: A Clonal Program For Eucalyptus Grandis (La Revista – Smurfit Carton de Colombia)

## Refereed Journals

### 23 Total


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Lambeth, C.C.; W.T. Gladstone and R.W. Stonecypher. 1982. Statistical precision of row and noncontiguous plots in genetic tests of loblolly pine. Silv. Gen. 32(1-2):24-28.

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Skoller, D.; F.E. Bridgwater and C.C. Lambeth. 1983. Fusiform rust resistance of select seedlots of loblolly pine in the lab, nursery and field. South. J. Appl. For. 7(4):198-203.

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Foster, S.F.; C.C. Lambeth and M.S. Greenwood. 1986. Growth of loblolly pine rooted cuttings compared with seedlings. Can. J. For. Res. 17:157-164.

Williams, C.G. and C.C. Lambeth. 1989. Bole straightness measurement for advanced-generation loblolly pine genetic tests. Silv. Gen. 38(5-6):212-216.

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Ladrach, W.E. and C.C. Lambeth. 1991. Growth and heritability estimates in 7-year-old *Pinus patula* progeny tests. Silv. Gen. 40(5/6):169-173.

Li, Bailian; C.G. Williams, W. Carlson, C.A. Harrington and C.C. Lambeth. 1992. Gain efficiency in short-term testing: experimental results. Can. J. For. Res. 22(3):290-297.

Dvorak, W.S.; C.C. Lambeth and B. Li. 1993. Genetic and site effects on stem breakage in *Pinus tecunumanii*. New For. 7(3):237-253.

Lowery, R.F.; C.C. Lambeth, M. Endo and M. Kane. 1993. Vegetation management in tropical forests. Can. J. For. Res. 23(10):2006-2014.

Wright, J.A.; L.F. Osorio and C.C. Lambeth. 1993. Development of a tree improvement program with *Pinus maximinoi* in Colombia. For. Ecol. and Mgmt. 62:313-322.

Lambeth, C.C.; M. Endo and J. Wright. 1994. Genetic analysis of 16 clonal trials of *Eucalyptus grandis* and comparisons with seedling checks. For. Sci. 40(3):397-411.

Lambeth, C.C. and R. McCullough. 1997. Genetic diversity in commercial forest tree plantations. (Invited paper in Proc. S. Reg. Inf. Exch. Group, July 1994). Can. J. For. Res. 27(3):409-414.

Frampton, L.J.; B. Goldfarb, S.E. Surles and C.C. Lambeth. 1999. Nursery rooting and growth of loblolly pine cuttings: Effects of rooting solution and full-sib family. South. J. Appl. For. 23(2):108-116.

Lambeth, C.C. 2000. Realized genetic gains for first generation improved loblolly pine in 45 tests in coastal North Carolina. S. J. Appl. For. 24(3):140-144.

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# Report on an Evaluation of Alternative Pine Breeding Strategies

**By**
**SELECT Breeding Services (CSIRO)**

**(Ian Purvis, John Henshall )**
January 2002

# Evaluation of Alternative Pine Breeding Strategies

**1. OBJECTIVES:** ***To evaluate, using stochastic simulation, the predicted genetic gains that would arise from pine breeding programs using a range of breeding strategies.***

## 2. PINE BREEDING STRATEGIES

The genetic gains from using three alternative breeding strategies were modelled using stochastic simulation techniques. These techniques take account of probability and sampling variation and thus generate information about the likely variance of outcomes

The five pine breeding strategies that were evaluated were as follows:

### *Strategy 1. Full-Sib Breeding System (Full-sib)*

40 parents mated at random to create 80 families (four crosses per parent in a circular mating system).

- Parameter estimates:
- → GCA of 40 parents,
  → SCA.
- Index selection:
- → $I = P_{individual} + b_1SCA_{family} + b_2GCA_{female} + b_3GCA_{male}$

### *Strategy 2. Polymix(PMX) Breeding System (PMX)*

- 40 parents mated with bulked pollen from the same 40 parents.
- Parameter estimates:
- → GCA of 40 parents as females,
  → No SCA.
- Index selection:
- → $I = P_{individual} + b_1GCA_{female}$

### *Strategies 3 and 4. Polymix and Limited Pedigree Assignment ( PMX_LPn )*

- 40 parents mated with bulked pollen from the same 40 parents,
  Stage-1: Top 100 (PMX_LP100) or 400 (PMX_LP400) individuals selected on
  $I = P_{individual} + b_1GCA_{female}$
  Stage-2: Selected individuals are fingerprinted.

  Final individual selection on: $I = P_{individual} + b_1SCA_{family} + b_2GCA_{female} + b_3GCA_{male}$

- Parameter estimates:
- → GCA of 40 parents as females,
  → Some SCA.

### *Strategy 5. Polymix and Full Pedigree Assignment (PMX_FPA)*

- 40 parents mated with bulked pollen from the same 40 parents,
  All individuals are fingerprinted,
  Individual selection on $I = P_{individual} + b_1SCA_{family} + b_2GCA_{female} + b_3GCA_{male}$

- Parameter estimates:
  → GCA of 40 parents as females and males,
  → SCA.

## 3. MATERIALS AND METHODS

Genetic and phenotypic parameters for the trait analysed (Height Growth) are contained in Table 1.

## Table 1: Genetic Parameters for Tree Height

| Genetic parameters | Height Growth |
|---|---|
| Individual heritability | 0.20 |
| Population mean | 6.0 m |
| Phenotypic variance among individual trees ($\sigma^2_P$) | 0.56 m |
| Coefficient of variation | 12.5 % |
| GCA additive variance ($\sigma^2_A$) | 0.0293 m |
| SCA additive variance ($\sigma^2_{NA}$) | 0.0083 m |

## 3.1. Basis for Simulation

***3.1.1 Genetic Parameters:*** A finite locus model was used for the genetic components of variance. In a finite locus model, gene effects are simulated as the sum of the effects of many small genes, instead of as from a continuous distribution (eg Fernando, Stricker and Elston, 1994 ). Finite locus models are well suited to modelling dominance and epistatic effects, and hence can easily encompass models including inbreeding depression and combining ability.

The genome was modelled as 200 loci, each having alleles 1 and 2, each with effect vector (*-a, d, a*) for genotypes (1,1), (1,2) and (2,2). Each allele was given a frequency of 0.5 in the base population.

Inbreeding depression was applied using the formula $\partial = 2F\sum d\overline{pq}$, where $\partial$ is the inbreeding depression and $\overline{p}$ and $\overline{q}$ are the allele frequencies in the population (Falconer, 1989). This measure of inbreeding is absolute rather than the relative measure required by the rate of inbreeding depression of 4% for every 0.1 increase in F, however, with $\overline{p}$ and $\overline{q}$ both set to 0 0.5, $\sum d = 4.8$ equates to the appropriate change when the mean is 6.0. For 200 loci the value of $d$ is 0.024.

Given that $d = 0.024$, for allele frequencies of $p = q = 0.5$, the dominance variance is $(2pqd)^2 = 0.000144$ for each locus, giving a total $\sigma_d^2 = 0.0288$. As the total genetic variance was specified to be 0.112, this leaves $\sigma_a^2 = 0.0832$, or $2pqa^2 = 0.000416$ for each locus, giving $a = 0.0288$.

The vector of allele effects then is (*-a, d, a*) = (-0.0288, 0.024, 0.0288).

GCA and SCA variance are functions of F, additive and non-additive genetic variance and the selection history of the population (eg cross between divergent lines). As the variances have been determined above, the appropriate levels of SCA and GCA were obtained by simulating breeding cycles between the base population and the experimental population. The choice of 200 loci was also made to produce a dominance variance conducive to obtaining the required levels of SCA and GCA. This process is not without error. Repeated simulations with constant genetic parameters and population structure can produce amounts of SCA and GCA, which are quite different.

*3.1.2 **Population Structure***: A base population of 250 individuals was generated, and a second generation of 200 individuals bred from the base through random mating. From these 200 individuals, 40 were selected on the basis of phenotype to be the parents of the first generation of experimental progeny. It was assumed that female parent was known for these individuals, and no more than four progeny from any one female parent were allowed.

The 40 selected parents were mated according to the specified strategies, and for each strategy a crop of 7800 progeny were produced. EBV's were estimated for these as specified for each strategy, except that for indexes

including SCA the phenotypes and parents of all 7800 individuals were used in an animal model to produce BLUP estimates of EBV. This is equivalent to using an index of own phenotype, and mean phenotypes for SCA, $GCA_{female}$ and $GCA_{male}$. To evaluate the accuracy of the EBV's ten further rounds of selection were performed, each with 7800 progeny from 40 individuals selected from the previous round.

Two simulations were performed for each strategy, the first with no restriction at all on inbreeding, the second with limits on relationship for the 40 selected parents for each crop of 7800 progeny. The inbreeding restrictions were: for strategies 1, 3, 4 and 5, where both parents were known, no more than one full sib and no more than four progeny from any parent were eligible for selection; for case 2 where only female parent was known, no more than four progeny from any female were eligible for selection.

## 4. RESULTS

### *4.1.*

***Validation***: To check that variance components were as expected a series of populations were simulated and GCA variance and SCA variance estimated. A summary of these estimates is contained in table 2. The estimated SCA variance is close to that desired, while the estimated GCA variance is a little high.

**Table 2: Validation of rate of inbreeding depression, GCA variance and SCA variance.** [Estimates are means from the 200 founder populations]

| | **CGA variance (sum of male and female)** | **SCA variance** | **Ratio GCA/SCA** |
|---|---|---|---|
| **Desired** | 0.0293 | 0.0083 | 3.53 |
| **Realised** | 0.0341 (0.0004) | 0.0075 (0.0003) | 4.53 |

### *4.2. Response to selection*

For each selection strategy, for each of unrestricted and restricted inbreeding, 200 replicates of the simulation were performed. The results presented are means and standard errors of means for both response and inbreeding.

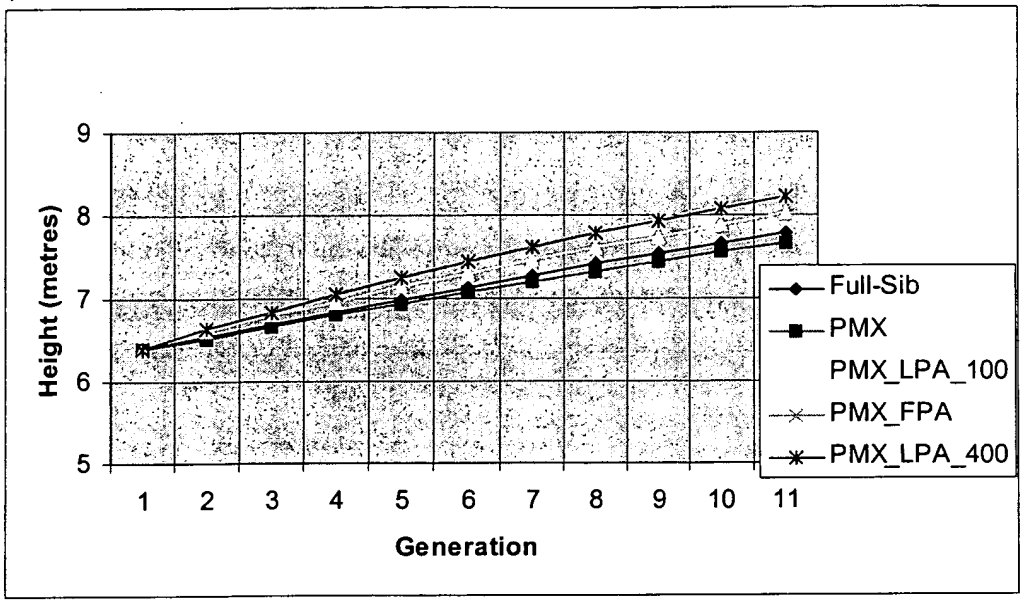
Figure 1 illustrates the relative gains made through the use of the 5 strategies with and without restrictions on inbreeding. With no restriction on inbreeding PMX_FPA and PMX_LPA400 produce almost identical response, and are superior to the other strategies, with PMX_LPA100 similar to Full-Sib, which is in turn superior to PMX. The means and standard errors in Table 3 suggest that by cycle 10, the differences between the groupings identified above are significant.

**Table 3. Mean (se) of tree height after 10 rounds of selection.**

| | Full-Sib | PMX | PMX_LPA100 | PMX_LPA400 | PMX_FPA |
|---|---|---|---|---|---|
| Unrestricted | 7.79 (0.01) | 7.66(0.01) | 8.02(0.01) | 8.23(0.01) | 8.26(0.01) |
| Restricted | 8.00(0.01) | 8.07(0.01) | 8.24(0.01) | 8.23(0.01) | 8.36(0.01) |

**Figure 1) Gains in Tree Height (metres) over 10 rounds of selection under Five Strategies**

**a) No Restrictions on Inbreeding**




**b) Restrictions on Inbreeding**

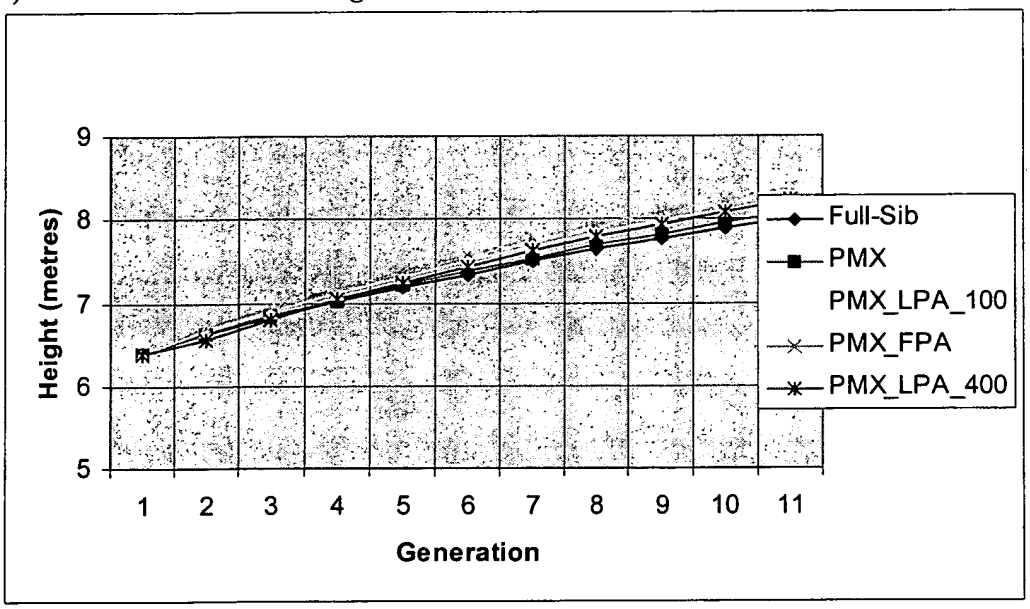
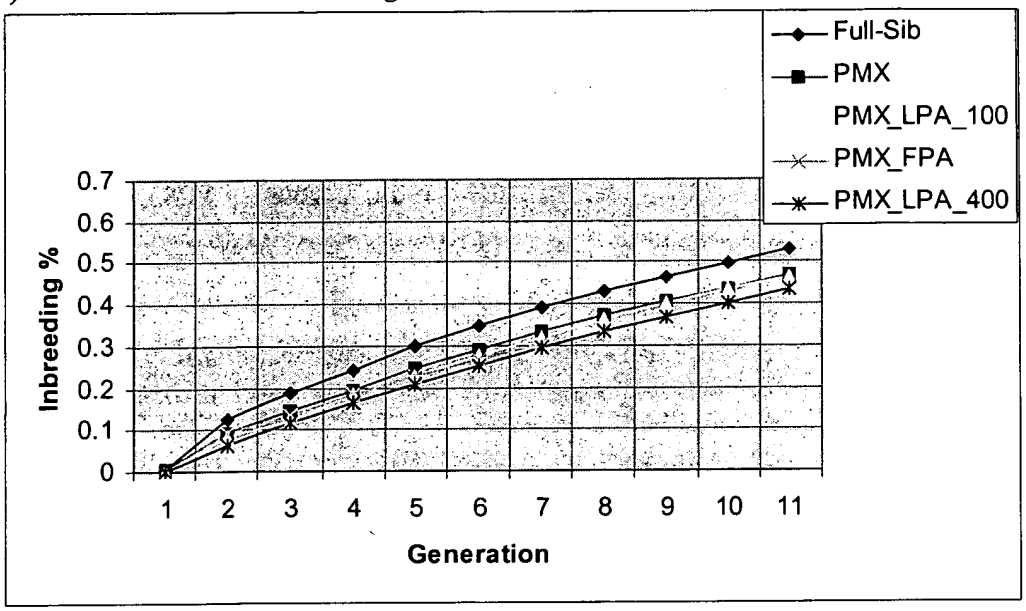


Figure 2 contains graphs of levels of inbreeding coefficients for each strategy over time. Of interest is the fact that the Full-Sib strategy has the highest levels of inbreeding when there is no restriction put in place. By contrast when the restrictions are implemented the effect is to reduce levels under the Full-Sib strategy to the lowest of all.

**Figure 2) Levels of Inbreeding over 10 rounds of Selection under five Strategies**
**a) No restrictions on Inbreeding**




b) **Restriction on Level of Inbreeding**

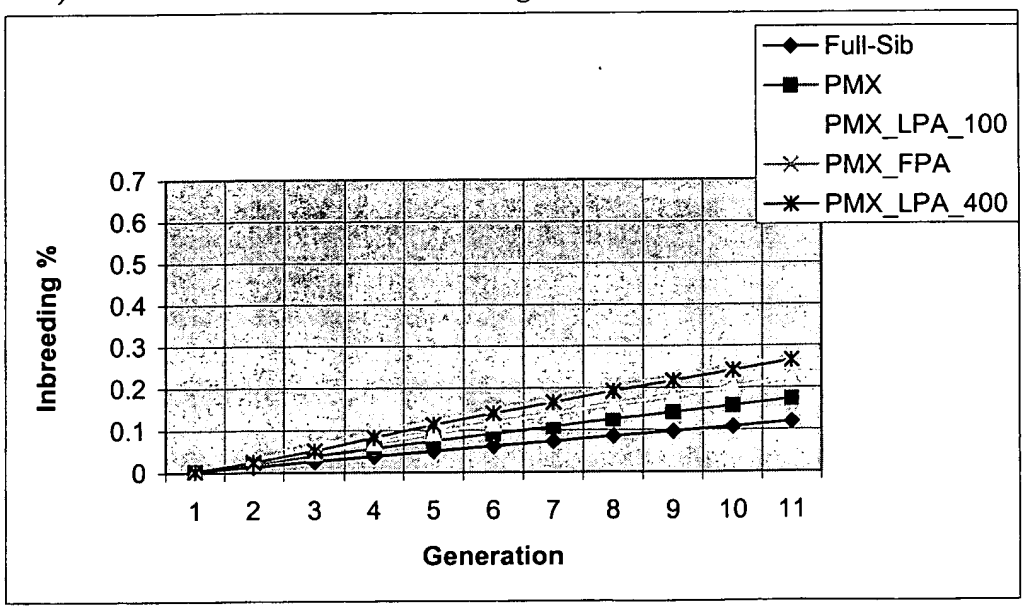


Table 4 clearly shows the cumulative effect of the inbreeding without restrictions by the 10th cycle and the clear effect of the restrictions, whilst also revealing slightly higher levels of inbreeding under the PMX_WPA strategies.

**Table 4. Mean (se) Inbreeding levels after 10 rounds of selection under the five selection strategies.**

| | Full-Sib | PMX | PMX_LPA100 | PMX_LPA400 | PMX_FPA |
|---|---|---|---|---|---|
| **Unrestricted** | 0.53(0.004) | 0.47(0.004) | 0.47(0.003) | 0.43(0.003) | 0.43(0.002) |
| **Restricted** | 0.12(0.0004) | 0.17(0.001) | 0.22(0.001) | 0.26(0.001) | 0.27(0.001) |

Accuracy of selection of individuals and parents in the initial and final cycles of selection are shown in Tables 5 and 6, respectively. The strategies employing parentage assignment are clearly more accurate. It is also noticeable that the accuracies have declined with time. This is likely due to the way in which the BLUP's and Indices were calculated. We did not include full pedigree information, which would have correctly accounted for selection. That is, by using only records and parentage of trees under consideration in any cycle, we were assuming "base" variance components for what were actually highly selected populations.

**Table 5. Accuracy of Selection of Individuals (Correlation between True Breeding Value and Estimated Breeding Value) under the five selection strategies.**

| | Full Sib | PMX | PMX_LPA100 | PMX_LPA400 | PMX_FPA |
|---|---|---|---|---|---|
| Initial Cycle | 0.460(0.003) | 0.452(0.001) | 0.508(0.001) | 0.529(0.002) | 0.611(0.002) |
| Final Cycle | 0.420(0.005) | 0.395(0.002) | 0.394(0.003) | 0.391(0.003) | 0.487(0.003) |

**Table 6. Accuracy of Selection of Parents (Correlation between True Breeding Value and Estimated Breeding Value) under the five selection strategies.**

| | Full Sib | PMX | PMX_LPA100 | PMX_LPA400 | PMX_FPA |
|---|---|---|---|---|---|
| Initial Cycle | 0.705(0.006) | 0.763(0.005) | 0.760(0.005) | 0.761(0.005) | 0.790(0.004) |
| Final Cycle | 0.509(0.011) | 0.644(0.008) | 0.607(0.008) | 0.583(0.008) | 0.601(0.009) |

## 5. Conclusions

On the basis of these simulations inbreeding restrictions clearly need to be applied in order to achieve optimal gains under all strategies. This is dependent on the rate of inbreeding depression being as specified. An added benefit of applying restrictions is that the general population has lower levels of inbreeding.

The polymix strategies are clearly the best strategies in the unrestricted method and also when inbreeding restrictions are applied. Of the PMX with parentage assignment strategies, the FPA gives the highest response for the trait under consideration. There is also the potential to increase gains even further under this strategy by reducing the inbreeding even further using mate allocation strategies.

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# TREE BREEDING METHODS

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# CONTENTS

# SUMMARY

Tree breeding practices are reviewed and related to the methods used in plant breeding. These methods include: mass selection (collection of open-pollinated seed from the best trees in forest stands), mass selection with progeny testing (open-pollinated seedling seed orchards), simple recurrent selection (untested clonal seed orchards and seed stands), recurrent selection for general combining ability (clonal seed orchards with progeny testing, second stage orchards and control-pollinated seedling seed orchards), hybrid varieties (biclonal orchards, interspecific and interracial hybrids), reciprocal recurrent selection, clonal selection and propagation, and clonal selection and propagation with clonal testing.

Expressions are given for predicting genetic gains from different tree breeding methods (mainly as developed by Namkoong *et al.* (1966), with additional expressions for methods they did not cover).

Actual data from a 14-year-old open-pollinated progeny test of *Pinus radiata* (height 67 ft) for bole straightness (heritability = .60), and diameter b.h. (heritability = .19) are used to calculate predicted genetic improvement in these characteristics for different breeding methods under a range of operationally realistic selection intensities. (Some additional assumptions are made about genetic variances beyond the information provided by the progeny test.) The time needed to produce the first improved planting stock, assuming a *P. radiata* time scale is also calculated.

Highest gain for straightness is predicted for a second-stage clonal orchard but mass production of cuttings of the best tested clones show higher gains in diameter, almost as high gains in straightness and production of improved planting stock in 11 years as compared with 28 years. Progeny tested clonal orchards, biclonal orchards and control-pollinated seedling orchards all gave lower gains that were of the same order. The main reason for the much higher gains for diameter among tested clones bulk propagated vegetatively was the large amount of non-additive genetic variance assumed for this trait. Untested clonal seed orchards and open-pollinated seedling seed orchards showed genetic improvement that was much lower than that of the clonal orchard with progeny testing (about $\frac{2}{3}$ for straightness and $\frac{1}{2}$ for diameter). The use of seed stands gave genetic improvement about half that of the untested clonal orchard, while the collection of open-pollinated seed from selected trees gave considerably less improvement than the seed stands. The gains predicted for the latter two methods however, were still appreciable.

# INTRODUCTION

Tree breeders have a tendency to consider methods of breeding and improvement of tree species in isolation from the general methods of improvement of crop plants and domestic animals. This is largely because of the length of time needed for most tree species to reach sexual maturity and then economic maturity. Thus a single cycle of breeding from initial selection to selection in the next generation takes a very long time and it is rare indeed to find discussion in tree breeding literature beyond the first stages of the breeding cycle. In New Zealand, exotic forests have the highest growth rates of the temperate regions of the world and as high or higher than many tropical areas. Because of this, breeding cycles are much shorter and an examination of long term or multi-cycle breeding methods has more relevance than elsewhere.

Tree improvement work has been active during the past 15 to 20 years in New Zealand. Initial emphasis was largely on selection and testing of species and provenances, and later an improvement programme for *P. radiata* was initiated with the aim of providing adequate supplies of improved seed from clonal seed orchards. The objective was to provide all future requirements of seed, which also would have the moderate degree of genetic improvement which can be expected from intensive field selection only. With the further development of breeding work in this species involving the realisation of extra genetic improvement by progeny testing, as well as the initiation of new breeding programmes with other species, it has become necessary to make a general evaluation of all possible breeding methods and their underlying theory so that the method most suitable for each situation can be adopted and so that future developments are soundly based. This pattern of development in tree improvement is found with minor variations in most countries where active tree improvement programmes exist.

This review examines methods of crop breeding as described by Allard (1960), and relates them to methods of tree breeding currently practised or advocated. A review of the comparative theoretical study by Namkoong *et al.* (1966) indicates the composition of genetic gain for some tree breeding methods and expressions are developed for several others. Finally, actual data from 14-year-old *P. radiata* are then used to make numerical comparisons of genetic gains in two traits of contrasting heritability using the different methods under optimum practicable intensities of selection.

# RELATION OF PLANT BREEDING METHODS, TO THOSE OF TREE BREEDING

In a consideration of breeding methods currently in use with crop plants and their application to tree breeding, the framework used by Allard (1960) is most suitable. From the point of view of the breeder, species can be divided into two groups; those which are mainly self-pollinating and those mainly cross-pollinating. This distinction determines what breeding methods are available for the species, as those applicable to the self-pollinators are distinct from those that can be used with cross-pollinators. All cross-pollinating plants (unless they have been purposely inbred) are highly heterozygous and inbreeding of these will usually result in loss of vigour and other adverse effects. Self-pollinated plants consist of collections of independently existing, homozygous inbred lines which show no loss of vigour in this state. The goals of breeding programmes in the two types involve the production of vigorous, fairly uniform, heterozygous offspring of the cross-pollinators and homozygous pure lines of the self-pollinators. Where asexual reproduction such as by vegetative propagation is possible additional breeding procedures can be used. The reproductive biology of the species usually determines its particular breeding procedure. For instance, the ability to tolerate some generations of selfing by maize, a naturally out-crossing species, has made possible the use of inbred lines and hybridisation.

The main difference between the material tree breeders and crop breeders have to deal with is that in most cases crop plants have undergone countless generations of selection so that the species is quite different in its characteristics and population structure from the original wild population. Again, because crop plants are frequently annuals, the numbers of generations of selection that have been undergone in the past and that can be undergone in the future are vastly greater than with tree species. Tree breeding is also a new science that has only been widely applied in the last two decades. Usually, tree breeders are dealing with unimproved species, frequently as natural populations. When they are grown as exotics as restricted portions of natural populations, they sometimes become hybridised with other populations in the process of introduction.

Among the species of interest for tree breeding, all conifers are out-crossing, essentially monoecious, wind-pollinated species with separate male and female flowers. Of other species of interest, most are out-crossing, frequently wind-pollinated, sometimes insect-pollinated. There are few species of tree breeding interest that are largely self-pollinated so this group will be omitted from further consideration.

For out-crossing species of crop plants, every plant is likely to be heterozygous and this heterozygosity must be maintained during the breeding programme or restored at the end. In crop plants there is a wide

variety of agencies and mechanisms controlling cross-pollination and also a big variation in the ease with which this can be controlled by the breeder. With tree species, for conifers the collection of pollen and isolation of female flowers are in most cases quite simple operations. Controlled pollination in eucalypts involving emasculation of bisexual capsulate flowers is more difficult but nevertheless quite feasible. Perhaps the greatest practical problems of controlled poliination involve access to the crowns of mature trees, and the long periods between pollination and seed harvest, e.g. of over two years in *P. radiata*. However, as grafting or production of rooted cuttings enables the mature tree crown to be propagated nearer ground level, and the propagules normally have earlier sexual maturity than seedlings, a partial solution to this difficulty is available. Nevertheless, controlled mating of trees is an expensive, time-consuming and laborious procedure which, when it must be used, adds several years to the breeding cycle.

The most important breeding methods used with cross-pollinating crop species, enumerated by Allard (*loc. cit.*), are:

(i) Mass selection
(ii) Backcross breeding
(iii) Hybridisation of inbred lines to form hybrid varieties
(iv) Recurrent selection

Each of these will now be discussed and their application to tree breeding indicated. In addition vegetative propagation can be used to multiply selected trees from natural populations, or to multiply any product of other breeding methods, and its role in tree improvement will also be discussed.

### (i) Mass Selection

With this method desirable individual plants are chosen and their open-pollinated seed collected and bulked without a progeny test to produce the following generation. Selection is thus based on female parent phenotype only, as there is no control over pollination and thus male parentage of the seed collected. This corresponds, in the forestry context, to collecting open-pollinated seed from the best trees in the forest and using this to establish further plantations from which, in turn, the best trees are selected and so on[1]. It should be pointed out that collection of seed from silviculturally thinned stands involves an element of recurrent selection (see below) improving male and female parentage. The effectiveness of this procedure is determined by the heritability of the trait involved and the intensity of selection. If heritability is high, accurate identification of the genotype of the female parent results and the offspring are appreciably improved. The purpose is to progressively increase the proportion of superior genotypes in the population over several generations and thus to raise the average population value for the trait.

Mass selection has been effective in modifying traits in crop plants that are easily seen or measured. It has also been effective in developing varieties

[1] Note that certain tree breeders term the selection of superior trees and propagation of these in orchards as "mass selection", though in this procedure male and female parentage of the resulting seed is select.

for special purposes and in changing the adaptation of varieties to fit them for new sites. With trees one could expect that mass selection for morphological traits such as bole straightness and branching habit would be effective. Similarly the adaptation of an exotic species would be assisted by some generations of mass selection. In actual fact, mass selection for bole straightness, branch morphology and vigour has proved effective in Queensland with *P. elliottii* var. *elliottii* (McWilliam and Florence, 1955). Selection of the best trees in the forest and collection of their open-pollinated seed resulted in an increase in acceptable stems per acre from 112 from routine seed, to 206; there was a similar increase from 4 to 28 "plus" stems per acre. There is evidence from provenance trials of several exotic species in New Zealand, e.g. *P. radiata*, that a few generations of natural selection in its new habitat with some mild silvicultural selection, is sufficient to improve the general performance and particularly survival and hardiness of the species considerably.

Mass selection has proved rather ineffective, at least in the short term, in modifying yields of well-adapted varieties or species of crop plants. This has presumably been due in the main to the low heritability of yield which would result largely through a progressive reduction in additive genetic variation in the face of previous generations of selection, either natural or artificial. Other causes would include uncontrolled pollination so that male parents are random, not select, and the fact that intense selection can reduce population size to the point that inbreeding occurs with consequent loss of vigour. Tree size is a character for which competition has imposed countless generations of natural selection, reducing genetic variability. Variation between populations, provenances or species is likely to be much greater and should be exploited.

Mass selection combined with progeny testing can help overcome some of the deficiencies of simple mass selection. In this case in crop plants open-pollinated progenies are grown from a portion of the seed of all individual selections and only the seed which gives rise to the best progenies is bulked and sown to form the following generation. This procedure bears some resemblance to the practice in tree breeding of establishing an "open-pollinated seedling seed orchard" (Wright and Bull, 1963; Shelbourne, 1962). Here, open-pollinated progenies of select trees are grown in a progeny test and later, on the basis of the measurements in the test, only the best trees in the best families are retained and seed is then collected from them. Such a process involves family selection (removal of poorer families) and in addition some within-family selection involving removal of all but the best individuals within families. The latter procedure cannot normally be utilised in mass selection with progeny testing of crop plants.

This procedure is also somewhat similar to the crop-breeding procedure of line breeding. Here, after several cycles of mass selection and progeny testing, the open-pollinated seed of a number of the best progeny-tested plants is bulked and planted in an isolated plot where random mating is allowed. Annual re-establishment of such a plot provides a continuing source of improved seed. Excessive inbreeding is avoided by ensuring that an adequate number of unrelated lines are involved.

It is proposed that a more practicable and economical method of establishing open-pollinated seedling orchards than has been previously suggested is to bulk open-pollinated seed of select trees, establish the orchard at close spacings and then thin selectively on the basis of phenotype alone. Such a method will enable large acreages of orchard to be cheaply established and, as will be shown later, can be expected to give nearly as much improvement as the progeny test type of orchard. To avoid risks of inbreeding in offspring of the orchard, large numbers (200 or more) of open-pollinated families should be used.

**(ii) Backcross Breeding**

The backcross method is particularly suitable in crop plants for transferring specific genes to a good variety which is deficient in one or a few characteristics. In this method recurrent backcrosses to the more desirable parent are made while selection is practised for the characters being transferred from the donor parent. It is important that sufficient plants of the heterozygous recurrent parent variety are used so that its characteristic features will be maintained in the backcross-improved products.

The prospects for the use of backcross breeding are rather limited with tree species mainly because a single sexual generation takes periods varying from about 4 to 20 years. However, in species with early sexual maturity, provided the trait to be transferred can be assessed at a very young age, backcross breeding might be applicable. The traits most suitable for transfer are those with high heritability and high economic importance such as disease resistance. If disease resistance is easy to transfer it may be controlled by a single locus and in turn will be subject to mutations of the pathogen.

**(iii) Hybrid Varieties**

Hybrid varieties depend for their superiority on the hybrid vigour or heterosis associated sometimes with $F_1$ hybrids. Inbred lines, clones, races, varieties or even species can be crossed to achieve the heterotic effect. Once a suitable hybrid combination has been identified the parental genotypes must be maintained without change so that the same hybrid can be produced repeatedly. This has limited the use of hybrid varieties in crop breeding to those species that can be selfed repeatedly to give homozygous inbred lines and also those species that can be propagated vegetatively. Homozygosity also gives rise to parental lines of consistent known combining ability, and because gametes are genetically quite uniform, gives hybrid offspring of considerable uniformity.

The greatest development of hybrid varieties has been with maize (corn) whose biology is well suited to this method of breeding. Desirable parents are selected in open-pollinated populations, selfed through several generations to produce homozygous inbred lines and finally these lines are crossed. In practice the yield of seed from a cross of two inbreds is so poor that usually the best single-crosses, as those are called, are themselves crossed to form a double-cross (or four-way cross) which is the commercially produced hybrid. It is important to realise that if this procedure is followed, with no selection at any stage and random crossing

of inbreds, the hybridisation will merely restore the vigour lost by selfing. The improvements in grain yields of over 20% that have been achieved have resulted because of selection of the original parents, selection between and within inbred lines and finally intense selection between hybrids. The latter is the critical operation that is responsible for the big improvement in yield associated with corn hybrids. Of the approximately 100,000 inbred lines developed by 1951 (Kiesselbach 1951) only about 60 have proved suitable in certain combinations, from which to develop commercial hybrids. Selection for double-cross yields by single-cross performance has been found to be quite satisfactory.

There is little possibility of using hybrid varieties of this type in tree breeding. Any adoption of the same techniques of crossing inbred lines used with corn would be impossibly slow, e.g. involving 42 years to take *P. radiata* through four generations of selfing, even supposing this is possible for more than a very few genotypes. In addition, the performance of hybrid varieties of this type is often quite specific to a given environment; and in forestry we must breed varieties adaptable to the uncontrolled conditions found in a forest.

Heterosis, however, could be utilised in three other possible ways with tree species: interspecific hybridisation, interracial hybridisation and utilisation of specific combining ability between individuals within a population.

Interspecific crossing is being used in a limited number of tree improvement programmes as a means of achieving genetic improvement not available by other methods. This usually takes the form of combining useful features of one species with those of another, and results in general intermediacy of characteristics for the hybrid, as well as hybrid vigour. In Korea the *P. rigida* x *P. taeda* cross provides a vigorous hybrid that performs better than either parent species (Hyun 1963); in California the cross *P. radiata* x *P. attenuata* extends the range of the *P. radiata* parent on to higher altitude, colder sites while preserving its valuable features (Stockwell and Righter, 1946); in Europe the larch hybrid *Larix decidua* x *L. leptolepis* is probably the best example in forestry of a well-proven heterotic interspecific hybrid. As a means of achieving a commercially useful hybrid involving production of hybrid seed on a large scale, interspecific hybridisation in tree breeding is not generally very useful because of low viable seed yields, and difficulties in obtaining good yields of exclusively hybrid seed. Hybridising orchards can be used to produce $F_1$ seed by planting selected clones of one species in a matrix of selected individuals of the other species, which act as pollen parents. The resulting seed will always contain some of the pure species and there are difficulties in matching flowering phenology of the clones of the two species. Interspecific hybridisation is more promising if it is associated with vegetative propagation by rooted cuttings. By using this technique the best individuals from interspecific crosses can be bulk-propagated from hybrid offspring, as is commonly done in poplar breeding.

Inter-racial crossing, that is, crossing of different races or populations of the same species, might be expected theoretically to provide hybrids with better balanced genetic constitutions than interspecific hybrids while

giving high yields of fertile seed. Provided the two races were genetically divergent, the resulting cross would be heterozygous and there would be a good chance of obtaining hybrid vigour. Few of such crosses are yet old enough to evaluate and experimentation with this method is continuing. The races chosen for hybridisation should be well adapted to the site on which the hybrid will be grown or alternatively, should be from two opposite extremes of native environment to the intended site. A hybrid of the latter type is likely to prove more physiologically versatile, though the former would be better adapted.

The use within a population of specific crosses between individuals which show high specific combining ability for vigour or any other characteristic is a method of possible application in tree breeding, though at the present stage of development this is unlikely to be adopted until after several generations of mass selection or recurrent selection (see below). As in the use of inbred lines with corn, large numbers of "hybrid" or specific combinations must be tested to identify the best crosses for the traits in question and which also show other features of desirable quality. The success of such a programme will depend on the existence of appreciable amounts of dominance genetic variance for a characteristic in the population in question. Very extensive crossing and testing programmes will be required to identify the heterotic combinations, and there is the further problem of mass producing the seed of a particular cross. Two-clone seed orchards might be one solution but it is not known yet how serious the effects of selfing would be under these conditions. Artificial pollination may be the only practicable answer. Because forestry demands genetic material that is quite variable as an insurance against catastrophe, several crosses would have to be produced and their seed bulked for planting.

Specific crosses could not be used as a continued cyclic method of breeding as selection and crossing within the offspring of such two-clone orchards would result in sib mating and inbreeding. If a further generation of two-clone orchards were initiated, it would be necessary to return to an improved population derived from recurrent selection, mass selection, or recurrent selection for General Combining Ability. An alternative procedure which has the advantage of allowing a continued and cumulative breeding programme to exploit heterosis is discussed below (reciprocal recurrent selection).

**(iv) Recurrent Selection**

There are four types of recurrent selection, depending on the way in which individuals with desirable traits are identified. These are *simple recurrent selection* (identification on parent phenotype), *recurrent selection for general combining ability* (on average offspring performance from several testers), *recurrent selection for specific combining ability* (on offspring performance after mating with a single tester) and *reciprocal recurrent selection.*

Historically, the ideas and theory of recurrent selection were developed in response to the realisation that in corn breeding selecting within and between inbred lines and finally between different hybrid combinations

to find the best heterotic hybrids was an inefficient procedure. Raising the frequency of desirable gene combinations in the parent population could instead be done cumulatively, at the same time maintaining genetic variability and allowing favourable gene frequencies to be increased by genetic recombination.

*Simple recurrent selection*

Desirable genotypes are selected on the basis of their own phenotype and are intercrossed without restriction to produce populations for the second cycle of selection. This process is therefore different from mass selection in that both male and female parentage is select. Additional cycles of selection can be carried out as long as improvement continues. For given intensities of selection the genetic improvement expected will be double that for mass selection.

In tree breeding, the selection of plus trees, their propagation in a clonal seed orchard and the harvest of the seed represent a single cycle of simple recurrent selection. The clonal propagules are allowed to inter-pollinate randomly in the seed orchard and the seed produced is used to establish plantations from which the next cycle of selection is made. As no test crosses are made, simple recurrent selection, like mass selection, is only efficient for traits with heritability high enough to make accurate selection for a superior genotype from phenotypic evaluation. The other types of recurrent selection are more suitable for selecting for low heritability traits as they involve test crossing to measure combining ability.

*Recurrent selection for general combining ability (RS/GCA)*

This involves mating phenotypically selected individuals with a heterozygous tester stock of broad genetic base. In tree breeding practice this may involve:—

(i) crossing selections with a standard group of tester individuals
(ii) polycrossing select individuals with a pollen mix of several select individuals
(iii) collecting seed from open pollination of the select individual with the unselected population
(iv) using vegetative ramets of the select individual. (This provides adequate screening of individuals for GCA provided non-additive genetic effects are not very large.)

Measurement of the offspring when planted in a suitable field design will provide half-sib, full-sib, or clonal family averages which give estimates of general combining ability. The individuals with the highest GCA are then propagated (in corn by selfed seed or in trees by grafts or cuttings) and intercrossed. The seed from this intercrossing is bulked and used to form the population for the second cycle of selection.

This procedure corresponds to the use of a clonal seed orchard with progeny testing that is in general use in tree improvement programmes. Superior trees are selected on the basis of their own phenotype in the forest, and propagated vegetatively in an orchard or clonal archive. Seeds

are either collected from the select parent trees from open pollination, or alternatively from controlled crossing or polycrossing in the orchard to generate half sib families (polycrossing) or a group of full sib families, e.g. North Carolina Design II, 4 tester design (Roberds *et al.* 1967) from which GCA can be estimated. "Families" of clonal ramets can also be used for this purpose (Libby, 1964) though this has rarely been attempted with trees.

Where GCA is estimated from open pollination of the original parent trees in the forest, it must be assumed that the number of pollen parents is infinite, and that for different parent trees located in different parts of the forest the same pollen spectrum is being sampled. The larger the number of male parents that pollinate the female flowers on the parent tree, the better the chance that the pollen will be a good genetic sample of the population. The use of open-pollinated seed is thus liable to induce bias in estimation of GCA because of differences in pollen spectra and numbers of male parents represented. This method cannot be safely used where appreciable genetic differentiation of local populations in natural stands has occurred. It is worth noting that collection of quite large numbers of fruits from different parts of the tree, and for serotinous pine species, from different years' pollination will alleviate this problem to some extent. Similarly, collection of open-pollinated seed from single trees not growing in forest stands should be avoided. The big advantage of using open-pollinated testing is that the seed may be collected in the same year that the tree is selected and the progeny test can be planted about the same time as the clonal orchard.

Where polycrossing is used to generate half-sib families, a standard pollen mix of 10 or more clones can be used to pollinate all clones, or alternatively different pollen mixes can be used to pollinate different groups of clones. Where individuals in the pollen mix are being polycrossed their pollen should be left out of the mix.

Where full-sib test crossing is used, any number of tester males can be used though a minimum is four. This method provides information on specific combining ability as well but is the most costly in terms of controlled pollination and size of test (four or more families per select clone tested). Both polycrossing and test crossing usually involve the grafting of ramets of select clones and a wait of several years for these to produce male and female flowers and then control-pollinated seed.

If groups of ramets of the select clones are used to screen for general combining ability, because of no genetic variation within clones or between ramets of the same clone, relatively fewer cuttings are needed to estimate the clonal means than are seedlings to estimate half-sib or full-sib family means. Also the clonal test can be established at the same time as the orchard or could even be combined with it. If topophytic or physiological age effects are important with the rooted cuttings however, clonal testing for GCA would not be appropriate, and the gain from clonal testing would be much reduced.

The best portion of the original select clones can be retained in the orchard with the remainder being removed, or alternatively a new orchard can be established with these clones only. A point that is frequently over-

looked is that to achieve an appreciable increase in genetic improvement a rather large proportion of the clones must be rejected. The seed collected from the reconstituted orchard will produce stock for establishing plantations and these trees when sufficiently mature will form the populations for the next cycle of seleccion.

Recurrent selection for general combining ability represents the main breeding procedure that tree breeders are adopting or at least working towards. The very slow progress in comparison with agricultural crops is due firstly to the long sexual generation and secondly to the long "assessment generation", the time taken before meaningful evaluations of progeny can be made, which is usually the longer of the two. Prospects for reducing both these generation times are quite good. Some success has been achieved in accelerating initiation of flowering in some species. and as data on juvenile-mature correlations are built up, earlier evaluations of progeny will be possible for some characteristics.

Another procedure that comes under the designation "recurrent selection for GCA" is the controlled pollinated seedling seed orchard. Phenotypic selection is made in the forest in the normal way and controlled polycrossing is carried out either on the original parents, or on their grafts growing in a clonal archive a few years later. The polycross progenies are then planted in a progeny test which is later converted to a seed orchard after assessment of the progenies, by removing all but the best families and the poorer trees from the best families. In practice for silvicultural reasons, selection intensity within the progeny test is limited to a total of about 5%, or about 25% for family selection and 20% for within-family selection. An extra stage of selection is thus involved beyond that of the clonal orchard with progeny testing. Because of the operational difficulties of this method. low family and within-family selection intensities, as well as the possibility for considerable sib-mating in this orchard resulting in inbreeding, the relatively high gain expectation must be weighed carefully against the other disadvantages. Control polycross seed can be bulked, as was suggested for the open-pollinated seedling orchard and the test area thinned on the basis of individual phenotype alone. This again has many practical advantages and reduces gain only slightly, as will be shown later.

A further method of recurrent selection for GCA involves similar stages of selection to the control pollinated seedling orchard but these are more intense. Normal phenotypic selection is followed by clonal orchard establishment and matings are made to generate full-sib families in some design such as a partial diallel or 4 tester. Identification of the clones of highest GCA from the best crosses is made in the progeny test and the best individuals in these families are selected and propagated to form a "Second-Stage" clonal orchard as proposed by Roberds *et al.* (1968). The main requirement for satisfactory operation of this method is a large range of original phenotypic selections among which extensive crossing to generate a large number of unrelated families has been carried out. Improvement is then due to initial phenotypic selection, selection for general combining ability (family selection) and finally a quite intensive within-family selection.

*Recurrent selection for specific combining ability (RS/SCA)*

This was proposed by Hull (1945). Test crosses were to be made to a single homozygous tester to develop complementary strains that would achieve maximum heterozygosity and thus heterosis in hybrid combination. Repeated cycles of family selection on the basis of test cross performance would mean however that if some defect became apparent in the original tester much effort would have been wasted. In addition, this method would produce improved populations of very narrow genetic base and it is not considered satisfactory for tree improvement.

*Reciprocal recurrent selection (RRS)*

This is the fourth and final type of recurrent selection and was proposed by Comstock, Robinson, and Harvey (1949) as a procedure useful in selecting simultaneously for both GCA and SCA. Two heterozygous source populations (A and B) are required and should be as genetically divergent as possible, though not sexually isolated, and should have generally desirable growth and technological characteristics. A group of individuals from population A is selected and mated to a similar sample from population B. In the same way a select group from population B is test-crossed to population A. The individuals in each population which show highest general combining ability with the *other* population, based on the performance of the test crosses, are later mated to each other *within* populations and the resulting offspring of each population are used to make the second cycle of selection. It is necessary to use enough plants in each generation for intense reselection after the progeny tests, and to leave a sufficient number after this to avoid serious inbreeding. The populations developed by several cycles of RRS could be crossed at any stage and, depending on the amount of divergence in gene frequencies and thus heterozygosity achieved, the resulting hybrids will be more or less heterotic. This scheme will build up two populations that are high in GCA for the characteristics selected for, and, if inter-racial heterosis for characteristics such as yield does exist, these populations will become progressively more divergent in their gene frequencies so that maximum heterosis will result when they are crossed. In addition, the hybrid will be generally intermediate in characteristics between the two populations.

Comstock *et al.* (1949) compared theoretically the efficiency of this method with *recurrent selection for general combining ability* and *recurrent selection for specific combining ability* under three levels of dominance—partial, full, and overdominance. They concluded:

(i) if dominance is partial, RS/GCA and RRS are about equally efficient and both are superior to RS/SCA
(ii) if dominance is complete, the three methods are essentially equal
(iii) if overdominance exists, RRS and RS/SCA are equal and both are superior to RS/GCA.

These comparisons assumed no epistasis, two alleles per locus and linkage equilibrium. The presence of epistasis (non-allelic gene interactions) would favour RRS and RS/SCA and linkage disequilibrium would also favour RRS. This system of breeding shows some possible applications

in tree improvement. In programmes working with fast-grown exotics, two races (provenances) of a species each with equally good performance but from distant parts of the natural species range can be combined into a heterotic hybrid or a hybrid that will tolerate the contrasting environmental conditions of each race. Such a scheme would take no longer than a conventional one for production of progeny tested seed, but would increase the number of selections necessary as two populations must be maintained with an adequate genetic base. This would involve the following steps when applied to a tree species:

1. Select 200 best phenotypes each from provenances A and B. Propagate by cuttings or grafts in randomly mixed clonal archive, with each population isolated from the other. Also propagate all 400 clones in mixed hybridising orchard at close spacings.
2. Make controlled polycrosses on all clones using pollen mix of sample from population A on population B and vice versa. Collect seed, grow stock and plant in progeny test.
3. After 10 years or so evaluate all progenies and remove the poorer 80% of clones from each population archive. Allow interpollination within each population and collect open-pollinated seed to establish commercial plantations of each population. Remove the poorer 80% of clones from the hybridising orchard, and allow interpollination and collect mixed hybrid seed.
4. After 10 years or so make second cycle of selection of best phenotypes in commercial plantations of each population, as in 1 above and repeat test crossing, etc. Continue cycles of selection, test crossing, etc.

Natural hybridisation of the two populations in the hybridising orchard can occur at any time. Alternatively, manual pollination of clones in the single population archives by pollen collected from the other population would result in production of pure hybrid seed. Furthermore, provided the species involved was commercially propagatable by cuttings, any superior hybrid at any point in the scheme could be multiplied by this method (see below).

This scheme or variants of it could be initiated with any species provided two desirable but genetically divergent provenances existed. Little extra work need be involved as total selection intensities within each population could be adjusted to the same as would be used in a single population. The main prerequisites for such a scheme to be initiated would be the availability of stands of two genetically divergent populations (provenances) of sufficiently desirable nature to make improvement within these a satisfactory proposition. In addition, some data from preliminary crosses between such populations giving indications of heterosis for yield and satisfactory quality of the hybrids would be needed.

### (v) Vegetative Propagation and Clonal Selection

The use of vegetative propagation to produce commercial planting stock is not strictly a method of breeding, though it is normally associated with selection and achieves a considerable improvement in the species con-

cerned. Phenotypic selection combined with clonal testing with vegetative propagules and ultimate release of tested clones is a procedure by which considerable improvement of a species can be achieved within a single sexual generation. It is thus a useful adjunct to any breeding procedure in that clonal testing will enable precise evaluation of the genotypes to be made, and if propagation techniques are economic, will enable any superior genotype to be replicated indefinitely for commercial use.

Extensive use is made of this approach in horticulture and agriculture with high value crops like potatoes, strawberries and fruit trees which are all dependent on vegetatively propagated, single genotypes. Possible applications of this technique in forestry have been limited by the ability to root cuttings of tree species on a commercially economic scale, and particularly to root cuttings from trees old enough among which to make meaningful phenotypic selections. Forest tree planting stock, unlike fruit trees, must be mass produced cheaply. An additional use for rooted cuttings is in establishing clonal tests for general combining ability; even when rooted cuttings cannot be produced easily and cheaply this method of testing has many advantages over using families, but some disadvantages as well. Also, in conjunction with other genetic experiments, clonal tests can help in estimating non-additive genetic variance.

Rather few forest tree species have been propagated commercially by rooted cuttings. Poplars and poplar hybrids are an outstanding example of a tree genus that has probably reached a higher level of improvement than any other forest tree species. Rooted cuttings of *Cryptomeria japonica* of certain selected clones have been widely planted in Japan, and *Salix* spp. are usually multiplied by cuttings. Recent work on *P. radiata* (Thulin and Faulds, 1968) indicates that commercial propagation of this species by rooted cuttings is feasible, and in a further paper (Thulin, 1969) the operation of an integrated programme for the production of progeny tested seed and commercial production of cuttings of tested clones is described.

Once commercially applicable techniques of rooting cuttings of forest tree species have been developed, phenotypic selection and clonal testing of any superior individual, the product of any breeding method be it backcross breeding, interspecific hybridisation or even natural or induced mutation[1] can be multiplied and utilised by commercial forestry. Perhaps the greatest advantage of using clonal plantations at the present stage of development of forest tree breeding is the ability to effect more rapid and greater improvement in genetic quality of planting stock than could be achieved by any other breeding method during the first cycle of a breeding programme. It should be emphasised, however, that provided the whole population has been effectively examined, the gain from cycles of selection is not cumulative as in simple recurrent selection; further gains can only be made by selection from an improved population derived from selection and genetic recombination.

[1] After an initial enthusiasm for mutation breeding (the induction of gene and chromosomal mutations artificially to produce greater phenotypic variation), most plant breeders quickly lost interest in this method choosing to make use of generally abundant existing variability rather than to depend on haphazard mutations, mostly deleterious, that were produced by radiation.

# EXPRESSIONS FOR PREDICTING GENETIC GAINS

In the preceding review of plant breeding methods applicable to cross-pollinated species it was attempted to relate these to the tree breeding procedures at present in use. Comparisons between different procedures were of a qualitative nature. It is possible, provided estimates of certain population parameters are available, to make comparisons of predicted genetic gains from different breeding methods in quantitative genetic terms. The best method of comparison would be by an economic analysis in terms of money invested in breeding on a per acre basis, and the expected increase in income per acre at the end of the rotation by the improvement in quantity and quality of wood. This type of analysis is well beyond our present knowledge. At best, in planning a breeding programme at present we can try to maximise our predicted genetic gain for a given expenditure and this involves adjusting the amount of work and thus expenditure involved in different phases of the programme in relation to the amount of genetic gain predicted, e.g. the costs of selection against the costs of progeny testing.

Several authors have attempted to make theoretical comparisons of different tree breeding methods, e.g. Wright (1962), Wright and Bull (*loc. cit.*), Stern and Hattemer (1964), Johnssen (1964), Libby (1964, 1965), Namkoong (1965), Namkoong *et al.* (1966), and Squillace *et al.* (1967). It is unfortunate that some of these attempts contain errors and inconsistencies, e.g. Wright, Wright and Bull, and Stern and Hattemer. By far the best and most comprehensive account is by Namkoong *et al.* (1966). They review heritability and gain concepts, and derive expressions for predicting genetic gains under different methods of breeding. Their expressions for gain prediction will be reviewed and expressions will be given for genetic gain prediction for additional breeding procedures not covered in their paper.

To estimate genetic gain, provided a properly constructed and precisely estimated heritability is available for the trait in question, it is only necessary to determine the selection differential. Breeding programmes in forestry commonly involved several stages of selection, each having its own selection differential and heritability. Selection is usually on the basis of a fraction of the population saved, and so the selection differential is measured indirectly. If a normal distribution of values for a characteristic under selection, i.e. values from individual trees or family averages exists with a variance $\sigma_u^2$, the difference between the mean of the select group and the population mean, i.e. the selection differential, can be derived to be $i\sigma_u$ where i is the selection intensity. The values of i for various population sizes are given by Fisher and Yates (1953, Table 20), Falconer (1960, pp. 193–4), and Nanson (1967). Values of i for a population of greater than 50 are also shown in Appendix Table 1.

NETIC GAINS

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TABLE 1. SUMMARY OF PLANT BREEDING METHODS WITH EQUIVALENT TREE BREEDING PRACTICES

| Plant Breeding | Tree Breeding |
|---|---|
| 1. Mass selection | Collection of open-pollinated seed from selected trees, usually at relatively low selection intensities (0.1–5% of stand selected). |
| 2. Mass selection with progeny testing | Open-pollinated seedling seed orchard (also involves additional within half-sib family selection). |
| 3. Simple recurrent selection | (i) Clonal seed orchard.<br>(ii) Seed stand (seed production area). |
| 4. Recurrent selection for general combining ability | (i) Clonal seed orchard with progeny or clonal testing followed by roguing or establishment of new orchard.<br>(ii) Control-pollinated (polycross) seedling orchard (also involves additional within half-sib family selection).<br>(iii) Second stage clonal seed orchard (selection of best individuals from the highest GCA clones' families in progeny test, with establishment of clonal orchard). |
| 5. Reciprocal recurrent selection | Reciprocal recurrent selection—not yet in use but has possible application in combining RS (GCA) with interracial hybridisation. |
| 6. Hybrid varieties (utilisation of heterosis) | (i) No real equivalent currently in use in tree breeding (with inbred lines).<br>(ii) Heterosis may be utilised by:<br>(a) interspecific hybrids<br>(b) inter-racial hybrids<br>(c) specific combining ability—biclonal orchards |
| 7. Selection and mass vegetative propagation | Selection and afforestation with rooted cuttings—limited use so far with poplars, willows etc. |
| 8. Selection, and mass vegetative propagation of tested clones | Selection and afforestation with rooted cuttings of best tested clones. |

Predicted genetic gain is given by the expression

$$\Delta G = i\sigma_u b \qquad (1)$$

where b = heritability for the particular selection system[1]

$$= \frac{k\sigma_A^2}{\sigma_u^2}$$

Thus: $\Delta G = \frac{ik\sigma_A^2}{\sigma_u}$

Where $\sigma_A^2$ = additive genetic variance

k = fraction of the total additive genetic variance in the covariance of additive values for the particular relatives in question, e.g. $k = \frac{1}{4}$ for selection amongst half-sib families.

In the preceding review, several breeding methods having application in forestry were discussed and a summary of these with the equivalent tree breeding practices is given in Table 1. The expressions for expected genetic gain for these methods will now be compared.

[1] b has been referred to as the heritability for the particular selection system (Namkoong *et al.* 1966). However a more customary formulation of heritability is $h^2 = \frac{\sigma_A^2}{\sigma_u^2}$ where $\sigma_u^2$ is the phenotypic variance of the individual tree.

### (1) Mass Selection

Mass selection involves the selection of the best trees in forest stands and the collection of their open-pollinated seed.

$$\text{Predicted gain, } \Delta G = i\tfrac{1}{2}\frac{\sigma_A^2}{\sigma_1} \qquad (2)$$

where $k = \frac{1}{2}$ (one parent only is selected)

$\sigma_1^2$ = phenotypic variance of the forest stand
$= \sigma_w^2 + \sigma_p^2 + \sigma_{ge}^2 + \sigma_G^2$

$\sigma_w^2$ = the environmental error variance of the small plot or microsite. The total within-plot variance $\sigma_t^2$ contains $\sigma_w^2$ plus the remaining genetic variation depending on the family structure of the experiment.

$\sigma_p^2$ = The error variance of large plots, or the failure of genotypes to behave in the same way between macrosites (commonly estimated in experiments as a family x replication interaction).

$\sigma_{ge}^2$ = The genotype x environmental or macrosite interaction, or error variance caused by the failure of genotypes to behave in relatively the same way in different environments.

$\sigma_G^2$ = Total genetic variance.

### (2) Mass Selection and Progeny Testing

Mass selection and progeny testing corresponds most closely to the open-pollinated seedling seed orchard. This involves collecting open-pollinated seed from select trees and planting half-sib progenies in a test design which is later thinned to the best trees in the best families.

$$\Delta G = i_1\frac{\frac{1}{2}\sigma_A^2}{\sigma_1} + i_2\frac{\frac{1}{4}\sigma_{A'}^2}{\sigma_2} + i_3\frac{\frac{3}{4}\sigma_{A'}^2}{\sigma_3} \qquad (3)$$

The gain $i_2\frac{\frac{1}{4}\sigma_{A'}^2}{\sigma_2}$ is due to selection of the best families and the element $i_3\frac{\frac{3}{4}\sigma_{A'}^2}{\sigma_3}$ corresponds to additional selection of the best trees within the best families. $i_1$, $i_2$ and $i_3$ are selection intensities determined by proportions of individuals in the whole population, of families amongst those originally selected and of individuals within families that were saved.

$\sigma_1^2$ = phenotypic variance in population, as equation (2)

$\sigma_2^2 = \frac{\sigma_t^2}{nre} + \frac{\sigma_p^2}{re} + \frac{\sigma_{ge}^2}{e} + \frac{1}{4}\sigma_A^2$. (Variance of half-sib family means)

$\sigma_3^2 = \sigma_w^2 + \sigma_p^2 + \sigma_{ge}^2 + \frac{3}{4}\sigma_A^2 + \sigma_D^2$ (within half-sib family variance)

$= \sigma_t^2 + \sigma_p^2 + \sigma_{ge}^2$

n = number of trees per plot

r = number of replications/location

e = number of locations

$\sigma_D^2$ = dominance genetic variance

The double prime on $\sigma_A^2$ indicates that this variance represents that in the population after selection, mating and thus genetic recombination have occurred. It can be assumed that $\sigma_A^2 = \sigma_{A'}^2$, as genetic variance will be regenerated after recombination. $\sigma_{A'}^2$ however represents the additive genetic variance left in the restricted, selected portion of the original population. It is less than $\sigma_A^2$ by an amount depending on heritability and intensity of selection.

$\sigma_{A'}^2 = \sigma_A^2 (1 - \beta\nu')$, where $\beta = \sigma_A^2/\sigma_1^2$ and $\nu'$ is a variable dependent on $i_1$ and is tabulated by Finney (1956) and shown in Appendix, Table 2.

Alternatively open-pollinated seed from plus trees may be bulked, the offspring planted at close spacing, and thinned later on the basis of individual phenotype only.

$$\Delta G = i_1 \frac{\frac{1}{2}\sigma_A^2}{\sigma_1} + \frac{i_4 \sigma_{A''}^2}{\sigma_4} \qquad (4)$$

Where: $i_4$ = combined intensity of $i_2$ and $i_3$ above; i.e. the proportion left after thinning.

$\sigma_4^2 = \sigma_w^2 + \sigma_p^2 + \sigma_{ge}^2 + \frac{3}{4}\sigma_{A'}^2 + \frac{1}{4}\sigma_{A'}^2 \simeq \sigma_3^2$

$\sigma_{A''}^2 = \frac{3}{4}\sigma_{A'}^2 + \frac{1}{4}\sigma_{A'}^2$

### (3) Simple Recurrent Selection

Simple recurrent selection is equivalent to a clonal seed orchard without progeny testing, or to a seed stand or seed production area where an existing stand has been thinned to the best trees and seed is collected from these. In both cases random interpollination of selected parents produces seed.

$$\Delta G = i_1 \frac{\sigma_A^2}{\sigma_1} \qquad (5)$$

where $\sigma_1^2$ = phenotypic variance as in equation (2).

Note: k = 1

**(4) Recurrent Selection for General Combining Ability**

This method involves phenotypic selection (as in (5) above) followed by either reselection amongst select individuals on their average test cross or polycross progeny performance (clonal seed orchard with progeny test reselection), or the selection of new select parents from the highest GCA families in the test. The latter procedure includes the control-pollinated seedling seed orchard, and the second stage clonal seed orchard where the new select parents are propagated vegetatively.

*(a) Clonal seed orchard with reselection by:*

*(i) Control-pollinated North Carolina Design II test*

GCA can be estimated by making control-pollinated test crosses of each select clone to a standard series of testers (e.g. Roberds *et al.*, 1967).

Predicted gain is given by:

$$\Delta G = i_1 \frac{\sigma_A^2}{\sigma_1} + 2\, i_2 \frac{\frac{1}{4}\sigma_{A'}^2}{\sigma_2} \qquad (6)$$

where $i_1$ and $i_2$ represent selection intensities of individual and progeny test selection.

$\sigma_1^2$ = phenotypic variance as in equation (2).

$$\sigma_2^2 = \frac{\sigma_t^2}{nre} + \frac{\sigma_p^2}{re} + \frac{\sigma_{ge}^2}{e} + \frac{\sigma_{mf}^2}{4} + \frac{\sigma_{A'}^2}{4}$$

$$= \frac{(\sigma_w^2 + \frac{3}{4}\sigma_{A'}^2 + \frac{15}{16}\sigma_D^2)}{nre} + \frac{\sigma_p^2}{re} + \frac{\sigma_{ge}^2}{e} + \frac{\sigma_D^2}{16} + \frac{\sigma_{A'}^2}{4}$$

$\sigma_{mf}^2$ = male x female interaction

$= \frac{1}{4}\sigma_D^{2\prime}$

The gain is thus in two parts; $i_1 \frac{\sigma_A^2}{\sigma_1}$ from phenotypic selection, and $2\, i_2 \frac{\frac{1}{4}\sigma_{A'}^2}{\sigma_2}$ from progeny test reselection. It should be noted that the single prime on $\sigma_A^2$ refers to reduced genetic variance in the restricted portion of the original population that is being progeny-tested, and that the gain from progeny testing is multiplied by two as both future parents of seed orchard seed are being reselected (on the basis of their progeny performance).

*(ii) Polycross or open-pollinated test*

GCA can also be estimated by controlled pollination of all select clones using a standard pollen mix of some of them. If the number of clones in the pollen mix is large, the only component of equation (6) which will change will be $\sigma_2$. The $\sigma_{mf}^2$ component of $\sigma_2^2$ is divided by

[1] Note that when considering genetic variances within and between families, epistatic interactions are disregarded.

the number of tester clones; this component can be assumed to be zero if more than 10 testers are used.

### (iii) *Clonal test for GCA*

GCA can also be estimated using vegetative propagules of the select parent such as rooted cuttings, as Libby (1964) indicated. Libby showed that the additional genetic gain from clonal selection is always considerably greater than that expected from full-sib or half-sib family selection. He compared the ratios of family selection to individual selection gains for half-sib, full-sib, and clonal "families". This is not the same as a progeny test situation where initial phenotypic selection is followed by subsequent reselection on the basis of a progeny test. Libby also assumed that topophytic or physiological age effects on the growth of the cuttings were absent. Assuming that there is perfect correlation between gene effects of seedlings and cuttings:

$$\Delta G = i_1 \frac{\sigma_A^2}{\sigma_1} + i_2 \frac{\sigma_{A'}^2}{\sigma_2} \tag{7}$$

where $\sigma_1^2$ = phenotypic variance as in equation (2)

$$\sigma_2^2 = \frac{\sigma_w^2}{nre} + \frac{\sigma_p^2}{re} + \frac{\sigma_{ge}^2}{e} + (\sigma_{A'}^2 + \sigma_{D'}^2 + \sigma_{I'}^2) + \sigma_C^2$$

$\sigma_{A'}^2$, $\sigma_{D'}^2$ and $\sigma_{I'}^2$ = additive, dominance and epistatic variance in select portion of population = $\sigma_{G'}^2$

$\sigma_C^2$ = variance of "c" effects which are the effects due to vegetative propagation that result in non-equivalence of clonal variance, $\sigma_{C_1}^2$, and total genetic variance, $\sigma_G^2$. This can best be illustrated by the well-known difference in morphology of young cuttings or grafts of pines from young seedlings. Propagation has caused an effect of increasing the apparent age of the cutting. This effect varies from clone to clone with a variance $\sigma_C^2$.

### (b) *Control-pollinated seedling seed orchard*

A further procedure advocated in tree breeding that is a variant of "Recurrent selection for GCA" is the control-pollinated seedling seed orchard. After phenotypic selection of plus trees, controlled polycrossing using a mixture of select tree pollen is carried out either on the parent tree (usually impracticable) or on its vegetative propagules.

The polycross half-sib families are planted in a test that is later thinned on the basis of measurements to leave the best trees in the best families.

$$\Delta G = i_1 \frac{\sigma_A^2}{\sigma_1} + i_2 \frac{\frac{1}{4}\sigma_{A'}^2}{\sigma_2} + i_3 \frac{\frac{3}{4}\sigma_{A'}^2}{\sigma_3} \tag{8}$$

where $\sigma_1$, $\sigma_2$ and $\sigma_3$ are the same as in equation (3) (open-pollinated seedling orchard).

Note that the gain from phenotypic individual tree selection is twice that in the open-pollinated seedling orchard.

The three components of the gain realised from the products of the orchard thus represent phenotypic selection $i_1\frac{\sigma_A^2}{\sigma_1}$, a half-sib family selection for GCA $i_2\frac{\frac{1}{4}\sigma_{A'}^2}{\sigma_2}$ and within family selection $i_3\frac{\frac{3}{4}\sigma_{A''}^2}{\sigma_3}$. Each stage of selection has its own selection intensity, of which $i_2$ and $i_3$ are limited by the silvicultural problems of thinning the test area.

Alternatively, as in the open-pollinated seedling orchard, polycross seed may be bulked, and the progenies thinned on the basis of phenotype only.

In this case:

$$\Delta G = i_1\frac{\sigma_A^2}{\sigma_1} + i_4\frac{\sigma_{A'''}^2}{\sigma_4} \qquad (9)$$

see equation 4 for $\sigma_{A'''}^2$, $\sigma_4$, etc.

A further variant of this procedure involves instead of thinning the test, propagating the select individuals in a new "second stage" clonal orchard. The expression for expected gain would be the same as equation (8) if polycrossing were used and would be as follows where full-sib families were involved:

$$\Delta G = i_1\frac{\sigma_A^2}{\sigma_1} + i_2\frac{\frac{1}{2}\sigma_{A'}^2}{\sigma_2} + i_3\frac{\frac{1}{2}\sigma_{A''}^2}{\sigma_3} \qquad (10)$$

In comparison with the control-pollinated orchard, this provides scope for increasing $i_2$ and $i_3$ at the expense of extending the time needed to produce improved seed.

Expressions for $\sigma_2^2$ and $\sigma_3^2$ shown for equation (3) will be modified to each include $\frac{1}{2}\sigma_{A'}^2$ and $\frac{1}{2}\sigma_{A''}^2$ respectively. The possibilities for intense family and within family selection are only limited by the number of families in the test and the number of trees per family.

### (5) Utilisation of Specific Combining Ability—Biclonal Orchard

Perhaps the easiest way for tree breeders to make use of heterosis that has been a foundation for the hybrid varieties of plant breeding, is to exploit specific combining ability shown by individual cross combinations. The testing of considerable numbers of controlled crosses, arranged in a design to sample some of the possible combinations between the groups of phenotypically selected trees, could be followed by the setting up of several two-clone orchards.

$$G = i_1\frac{\sigma_A^2}{\sigma_1} + i_2\frac{\frac{1}{2}\sigma_{A'}^2}{\sigma_2} + i_2\frac{\frac{1}{4}\sigma_D^2}{\sigma_2} \qquad (11)$$

where $i_1$ = intensity of individual phenotypic selection
$i_2$ = intensity of selection among full-sib families

$\sigma_1^2$ = phenotypic variance as in equation (2)

$$\sigma_2^2 = \frac{\sigma_t^2}{nre} + \frac{\sigma_p^2}{re} + \frac{\sigma_{ge}^2}{e} + \frac{\sigma_D^2}{4} + \frac{\sigma_A^2}{2}$$

There are thus three elements of gain; the first due to individual phenotypic selection is the same as in other procedures; the second is that due to selection of the best full-sib families, and the third element is again based on the full-sib family but is dependent on dominance variance. If this variance is large, the extra gain from this approach will be considerable.

### (6) Reciprocal Recurrent Selection

This method involving parallel phenotypic selection in two populations with parallel test crossing of select individuals in each population to a select sample from the other population, allows the cumulative improvement and moulding of two populations so that they have increasingly divergent gene frequencies and thus increased heterosis on crossing at subsequent stages in the programme. An expression for the genetic gain achieved by this system cannot be given. It will be made up in the same way as equation (11) except that non-additive gene effects are likely to be much greater when chromosome sets of widely differing origin are brought together.

### (7) Selection and Mass Vegetative Propagation

Phenotypic selection followed by bulk vegetative propagation of select tree planting stock can provide considerable genetic improvement. At the present stage of development in tree breeding its most important application is phenotypic selection in unimproved forest stands.

A complicating factor with many species is that the process of propagation maintains the adult characteristics of the ortet in the propagule. For instance, in many pines cuttings or grafts of older trees show the morphology of the upper crown of the parent tree. This effect is represented by an additional constant, $\bar{c}$, in the prediction of genetic gain thus:

$$\Delta G = \bar{c} + i_1 \frac{\sigma_G^2}{\sigma_1} \qquad (12)$$

where $\sigma_G^2 = \sigma_A^2 + \sigma_D^2 + \sigma_I^2$

= total genetic variance

$\sigma_I^2$ = epistatic variance

$\bar{c}$ = average propagation effect[1] in using rooted cuttings rather than seedlings

$\sigma_1^2$ = phenotypic variance as in equation (2)

[1] Different gene effects may be involved in the control of "adult" cutting versus "juvenile" seedling traits. The present approach to the problem is to ignore this and assume that vegetative propagation of physiologically "older" material merely produces an average modifying effect.

Depending on the size of the non-additive genetic variance ($\sigma^2_D$ and $\sigma^2_I$), gains will be greater than from simple recurrent selection. It is probable that for certain species the average propagation effect, $\bar{c}$, may be greater than the genetic gain. It could be in a negative direction cancelling the effects of genetic improvement, though there are indications that at least in pine species the effects are supplementary to genetic gain for most morphological traits.

**(8) Selection, and Mass Vegetative Propagation of Best Tested Clones**

If clonal tests of rooted cuttings are established after phenotypic selection, and a further reselection is made among the select clones on their test performance, an additional gain can be obtained.

$$\Delta G = \bar{c} + i_1 \frac{\sigma^2_G}{\sigma_1} + i_2 \frac{(\sigma^2_{G'} + \sigma^2_C)}{\sigma_2} \qquad (13)$$

where $i_2$ = selection intensity among phenotypically select clones

$\sigma^2_{G'}$ = reduced total genetic variance after phenotypic selection

$\sigma^2_C$ = the variance of "c" effects (these may be permanent like physiological age effects in pines or transitory due to size and condition of cutting material).

$$\sigma^2_2 = \frac{\sigma^2_w}{nre} + \frac{\sigma^2_p}{re} + \frac{\sigma^2_{ge}}{e} + \sigma^2_{G'} + \sigma^2_C$$

It can be seen that the variance of clonal means ($\sigma^2_2$) is much reduced, firstly by the process of multiplication of each clone and secondly by the reduction in $\sigma^2_G$ to $\sigma^2_{G'}$. With high broad sense heritabilities of, e.g. .80, this variance could be reduced to about one quarter of its original size with corresponding overriding importance of $\sigma^2_C$, the "c" effect variance. However it is not known at present what size $\sigma^2_C$ is likely to be. Where broad sense heritabilities are high (over .80) and where $\sigma^2_C$ is small, clonal testing is unlikely to be worthwhile. However where heritabilities are lower and/or $\sigma^2_C$ is higher, then clonal testing will most probably be effective in substantially increasing genetic gain. Where it is planned to only use limited numbers of the best clones for commercial propagation, clonal testing is an essential safeguard.

# COMPARISON OF PREDICTED GAINS USING RADIATA PINE DATA

## Introduction

It is possible to compare predicted genetic gains for any species from the different breeding methods discussed previously provided heritabilities (broad and narrow sense) have been estimated for the trait under comparison. Actual variance data from *Pinus radiata* grown in New Zealand is now used to make comparisons of predicted gains under a range of selection intensities that are operationally practicable.

Namkoong *et al.* (1966) made comparisons of genetic gains using simulated variances for traits of low and moderate narrow sense heritability (.05 and .20) and conditions of effective and ineffective testing, i.e. where the reducible variance in the progeny test is maximally or minimally reduced. These comparisons were made for five breeding methods: clonal orchard (without progeny test), clonal orchard with progeny test, open-pollinated seedling orchard, control-pollinated seedling orchard and two-clone orchard. They also considered the time taken to achieve gains, and tabulated operational values per year, representing the total units of gain accrued throughout the breeding cycle divided by number of years in the cycle. To make gain from different methods mathematically comparable, they restricted all methods to a total selection intensity corresponding to 1 in 1000.

In terms of total gain realised, they found that the control-pollinated seedling orchard was generally just the best under low and moderate heritability and under effective and ineffective testing, though the superiority over the gains by the clonal orchard with progeny testing was generally slight. When operational value per year was considered, the clonal orchard with progeny testing was just superior to the control-pollinated seedling orchard. The two-clone orchard, except under high heritability and highly effective testing, was generally slightly inferior to or about equal with the former two methods. Interestingly, total gains from the untested clonal orchards were much lower, but about the same as the open-pollinated seedling orchard. Under effective testing the open-pollinated orchard showed a considerably higher operational value per year than the untested clonal orchard.

Two traits of radiata pine, stem straightness and breast height diameter, with contrasting heritabilities, will be used here to compare gains from a wider range of breeding methods, including those involving rooted cuttings.

## Methods

Estimates of additive genetic variance, plot-to-plot and within-plot variance for diameter at breast height and bole straightness (subjectively rated 0 to 9) were available from an experiment involving 29 open-

pollinated families of 14-year-old *P. radiata* (M. H. Bannister, manuscript in preparation). There were no estimates for dominance variance (assumed zero for straightness and equal to additive variance for diameter) or for genotype × site interaction (assumed zero). The variance of "c" effects or propagation effects, namely the effect of vegetative propagation on the cutting's growth and morphology, was assumed to be one quarter of within-plot variance for both traits. Heritability (narrow-sense) was .60 for straightness and .19 for diameter; heritabilities (broad-sense) were .60 and .37 respectively. (See Appendix for details of variances and calculations.)

Predicted genetic gains, expressed as a percentage of the population mean before selection, were calculated using these parameters for different breeding methods with varying intensities of selection. When methods involved phenotypic and subsequent genotypic selection (based on test performance), a phenotypic selection intensity corresponding to the best tree in 1,000 was used, and the intensities of progeny test reselection were varied from 1 in 2 to 1 in 100.

It must be recognised that some of the extrapolations involved in prediction of improvement in diameter growth and to some extent straightness may break down in practice owing to the effects of intertree competition and to non-linearity of the subjective rating scale for straightness. This should not affect the ranking of gains for different methods and the effects of heritability on this ranking.

**Results and Discussion**

For the different breeding methods shown, genetic gains are expressed as percentages of the population means for straightness and diameter in Table 2*. The greatest gains in both high and low heritability traits (straightness and diameter) were predicted for method 6, the second stage clonal orchard (87% and 27%) and for method 10, clonal propagation and testing (76% and 45%). However, using *P. radiata* as a basis of comparison, the stock from method 6 could only be planted 28 years after the initial phenotypic selection (see "Time to first improved planting stock", Table 2) while that from method 10 could be planted after 11 years. To provide sufficient unrelated families in which to make selections for the second stage orchard (Method 6) it requires a very large number of crosses with several different series of testers or a large number of individuals in the polycross pollen mix.

Gains in straightness of 70% from Method 5, the clonal seed orchard with progeny testing, were about the same as for the control-pollinated seedling orchard, Method 7a and b (71% and 74%), and only a little lower than Method 10. The gain of 45% for diameter with clonal propagation and testing is much higher than for any other method mainly because this method utilises the considerable dominance variance assumed for this trait.

An examination of test gains relative to total gains in Table 2 reveals that any form of testing results in relatively more additional gain with a low than with a high heritability trait. Also, where dominance or epistatic

* On pages 30–31.

variance is appreciable (for diameter), clonal propagation is able to exploit the total genetic variance, in contrast to other methods where only the two-clone orchard is capable of utilising part of the dominance variance (reciprocal recurrent selection, not covered in this comparison, can also do this).

It should be emphasised that the gains from clonal selection, propagation and testing do not take into account the average propagation effect $\bar{c}$, which can either complement or detract from genetic gain. However the clonal test enables the net effect of gain and propagation effect to be observed and the best performing clones can be identified and mass-produced for forest planting.

The effectiveness of the clonal seed orchard with progeny testing (Method 5) depends very much on how progeny test reselection is applied. If an open-pollinated test (Method 5b) was established at the same time or earlier than the original orchard and then a new orchard established of the best clones later, it would be 22 years from initial selection on the *P. radiata* time scale before the first progeny-tested planting stock was available. If a control-pollinated test was used it would be 27 years or more. If, however, the first orchard was close-spaced, e.g. 6 x 6 feet and thinned progressively on the basis of test data to 20 x 20 feet or wider, progeny tested stock could be produced after 15 years. This would be expensive if grafts rather than cuttings were used.

Different progeny test methods (5a, b, c) do not materially affect gains from the tested orchards. Full-sib designs, e.g. Method 5a are more costly, and more than one family per clone is necessary. All control-pollinated designs including polycrossing are costly and delay test establishment. Open-pollination is cheap, involves no delays but is less precise in estimating general combining ability (GCA). The use of rooted cuttings to screen for GCA is an interesting alternative to polycross and open-pollinated stock that allows early test establishment and far fewer plants per tested clone due to low within-clone variation. When non-additive variance and topophysis or propagation effects are large, however, it would be unsatisfactory.

The control-pollinated seedling orchard (Method 7a) shows about the same gains as the progeny tested clonal orchard; gains are composed similarly but are lower than for the second stage clonal orchard because silvicultural difficulties in thinning the test area limit the intensity of family and within-family selection.

If, however, polycross families are bulked and individual phenotypic selection only is carried out in the bulked stand (Method 7b), the genetic gains for both high and low heritability traits are not seriously reduced. Such an orchard would be very much cheaper and easier to plant and thin than the control-pollinated seedling orchard. The main requirement for such an orchard would be a large number of clones selected (e.g. 200), and the use of several pollen mixes with 10 or more pollens each to avoid the possibility of many individuals in the resulting orchards being related.

Two-clone orchards (Method 8) differ little in expected gains from progeny-tested clonal orchards. For diameter where dominance variance was assumed equal to additive variance, genetic gain was predicted to be

TABLE 2. COMPARISON OF GENETIC GAINS FOR DIFFERENT BREEDING METHODS

| Method | Proportion of Population Saved 1 in: | | Straightness | | Diameter | | Time[3]* (years) to first improved planting stock |
|---|---|---|---|---|---|---|---|
| | | | Total Gain % | Test Gain % | Total Gain % | Test Gain % | |
| 1. Collection of open-pollinated seed from best trees in stand | $i_1$ | 5 | 9.9 | | 2.3 | | |
| | | 10 | 12.4 | | 2.9 | | |
| | | 20 | 14.6 | | 3.4 | | 1 |
| | | 100 | 18.9 | | 4.4 | | |
| 2. Seed stand/seed production area. | $i_1$ | 10 | 25.0 | | 5.8 | | |
| | | 20 | 29.2 | | 6.7 | | 5 |
| 3. (a) Open pollinated seedling seed orchard. Phenotypic selection; open-pollinated test thinned to best trees in best families. | $i_1$[1]* | 1,000 | 46.9 | 23.1 | 12.9 | 7.4 | 15 |
| | $i_2$ | 4 | | | | | |
| | $i_3$ | 5 | | | | | |
| (b) As for (a) but bulked and thinned on phenotype only. | $i_1$ | 1,000 | 50.0 | | 12.0 | | 15 |
| | $i_2$ | 20 | | | | | |
| 4. Clonal seed orchard. | $i_1$ | 100 | 37.7 | | 8.7 | | |
| | | 1,000 | 47.6 | | 11.0 | | 11 |
| | | 10,000 | 56.0 | | 12.9 | | |
| | | 100,000 | 63.4 | | 14.7 | | |
| 5. Clonal seed orchard with: ($i_1$ = 1 in 1,000; $i_2$ varies). | $i_2$ | 2 | 56.5 | 8.8 | 15.4 | 4.4 | |
| | | 5 | 63.1 | 15.4 | 18.7 | 7.7 | |
| | | 10 | 67.1 | 19.4 | 20.7 | 9.7 | |
| Reselection of clones on basis of test, by roguing or planting new orchard. (a) 4 tester N.C. Design II Control-pollinated test. | | 20 | 70.4 | 22.7 | 22.3 | 11.3 | 27 (new orchard) |
| (b) Polycross or open-pollinated progeny test. | $i_2$ | 2 | 56.5 | 8.8 | 15.9 | 4.9 | *O.P. test* |
| | | 5 | 63.1 | 15.4 | 19.6 | 8.5 | 15 (thin), 22 (new orchard) |
| | | 10 | 67.1 | 19.4 | 21.8 | 10.7 | *Polycross test* |
| | | 20 | 70.4 | 22.7 | 23.6 | 12.6 | 27 (new orchard) |
| (c) Clonal test (for general combining ability). | $i_2$ | 2 | 56.0 | 8.3 | 14.2 | 3.2 | |
| | | 5 | 62.2 | 14.5 | 16.6 | 5.6 | |
| | | 10 | 65.9 | 18.3 | 18.0 | 7.0 | 15 (thin), 22 (new orchard) |
| | | 20 | 69.0 | 21.4 | 19.2 | 8.2 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (a) 4 tester N.C. Design II Control-pollinated test. | | | | | | | |
| (b) Polycross or open-pollinated progeny test. | $i_2$ | 2 | 56.5 | 8.8 | 15.9 | 4.9 | *O.P. test* |
| | | 5 | 63.1 | 15.4 | 19.6 | 8.5 | 15 (thin), 22 (new orchard) |
| | | 10 | 67.1 | 19.4 | 21.8 | 10.7 | *Polycross test* |
| | | 20 | 70.4 | 22.7 | 23.6 | 12.6 | 27 (new orchard) |
| (c) Clonal test (for general combining ability). | $i_2$ | 2 | 56.0 | 8.3 | 14.2 | 3.2 | |
| | | 5 | 62.2 | 14.5 | 16.6 | 5.6 | |
| | | 10 | 65.9 | 18.3 | 18.0 | 7.0 | 15 (thin), 22 (new orchard) |
| | | 20 | 69.0 | 21.4 | 19.2 | 8.2 | |
| 6. "Second stage" clonal seed orchard; new plus trees selected in full-sib test from highest GCA clones. | $i_1$<br>$i_2$<br>$i_3$ | 1,000<br>20<br>100 | 87.0 | 39.4 | 27.4 | 16.4 | 28 |
| 7. (a) Control-pollinated seedling seed orchard. Phenotypic selection; polycross test thinned to best trees in best families. | $i_1$<br>$i_2$<br>$i_3$ | 1000<br>4<br>5 | 70.8 | 23.1 | 18.4 | 7.4 | 22 |
| (b) As for (a) but bulked, and thinned on phenotype only. | $i_1$<br>$i_2$ | 1,000<br>20 | 73.9 | | 17.5 | | 22 |
| 8. Two-clone orchards: ($i_1$ = 1 in 1,000; $i_2$ varies). Phenotypic selection; full-sib testing, best specific combiners in two-clone orchards. | $i_2$ | 2 | 54.2 | 6.5 | 15.8 | 4.8 | |
| | | 5 | 59.1 | 11.4 | 19.3 | 8.3 | |
| | | 10 | 62.0 | 14.4 | 21.5 | 10.5 | 27 |
| | | 20 | 64.5 | 16.9 | 23.3 | 12.3 | |
| | | 100 | 69.5 | 21.8 | 26.8 | 15.8 | |
| 9. Selection of plus trees and their commercial-scale vegetative propagation[2*] as forest planting stock. | $i_1$ | 100 | 37.7 | | 17.5 | | |
| | | 1,000 | 47.6 | | 22.0 | | 6 |
| | | 10,000 | 56.0 | | 25.9 | | |
| | | 100,000 | 63.4 | | 29.3 | | |
| 10. Selection of plus trees, and commercial-scale vegetative propagation[2*] of the best clones only. $i_1$ = 1 in 1,000; $i_2$ varies. | $i_2$ | 2 | 58.6 | 11.0 | 30.8 | 8.8 | |
| | | 5 | 66.9 | 19.2 | 37.4 | 15.3 | |
| | | 10 | 71.8 | 24.2 | 41.3 | 19.3 | 11 |
| | | 20 | 75.9 | 28.3 | 44.6 | 22.6 | |

*Footnotes:* [1*] $i_1$ = intensity of phenotypic selection
$i_2$ = intensity of between-family (or between-clone) selection
$i_3$ = intensity of within-family selection

[2*] Gains do not include $\bar{c}$, average effect of vegetative propagation

[3*] *P. radiata* time scale (years)

Selection = 1
Grafting and clonal orchard planting = 1
Age to first commercial seed for clonal orchard = 8
Seed to planting stock = 1
Age to first commercial seed from seedling orchard = 14
Control-pollinated test: Time to first pollination = 3
Time from pollination to seed = 2
Total time from initial selection to planting test = 8
Age of progeny test for main assessment = 10

23%, the same as for the clonal orchard and polycross test (Method 5b). Gains for straightness were lower than 5b as might be expected.

Untested clonal orchards[1] show gains much lower for both straightness and diameter than tested orchards, e.g. 11% compared with 22% for diameter, underlining the value of testing and reselection.

The open-pollinated seedling orchard, generally regarded as a low gain method, shows predicted gains as high as an untested clonal orchard (47% and 13% for straightness and diameter). This method could be useful where a low cost, simple improvement programme is needed, or where serious difficulties are encountered with vegetative propagation, provided that the species flowers early in its life-cycle and that characters selected for can be assessed early. As for the control-pollinated orchard there are many practical advantages and little if any loss of gain in relying entirely on phenotypic selection (Method 3b) instead of between and within family selection for the open-pollinated seedling orchard. Open-pollinated seed is usually available in quantity from 200 or more select trees and when bulked permits large acreages of orchard, planted at close spacings and later thinned selectively, to be established very cheaply. Predicted gains of 50% for straightness and 12% for diameter show little difference from gains from Method 3a.

The use of rooted cuttings of untested select trees (Method 9) shows gains similar to the untested orchard for straightness and as high as the tested clonal orchard for diameter. This method has the great merit that rapidly increasing quantities of improved stock are available within a year of initial selection. With 500 clones selected, six years would be necessary to build up to an annual production of 300,000 rooted cuttings in *P. radiata* (Thulin 1969). It should of course be emphasised that continuing gains from this method and Method 10 require the provision of improved populations of seedlings for selection by methods 1 to 8.

As interim methods of tree breeding, gains from seed stands and seed collection from selected trees in the forest show that really worthwhile improvement can be made, e.g. by restricting seed collection to the best tree in 20, a gain of 15% in straightness is predicted compared with 48% from an untested clonal orchard; or a seed stand selectively thinned to 10% of original stocking could realise a 25% improvement in straightness. The important features of these methods are their low cost and early production of improved stock.

Something frequently not fully appreciated by tree breeders is the logarithmic relationship between selection intensity (proportion saved) and genetic gain. The gains from the clonal orchard (Method 4) show that to increase gain in diameter from 11.0% to 12.9%, an increase of 1.9% (contrast possible gain of 24% shown in Table 1 with progeny testing), the work and thus cost of plus tree selection must be increased tenfold. The breeder should therefore know the relative costs of selection and of establishing and assessing progeny or clonal tests so that by adjusting the proportion of expenditure on each he can maximise his genetic gain for a given outlay.

[1] An actual improvement of 63% in straightness and 8% in diameter was realised for this method in a *P. radiata* control-pollinated progeny test aged 10 years, height 50 feet. (Shelbourne 1969.)

These numerical comparisons of predicted gains are of limited value in a comparison of breeding methods. Other important factors are the length of the period before improved stock is available, the relative costs of different methods and stages of each method and the practical realities and problems of a particular situation. Generally the breeder is well advised to attempt maximum gains in any cycle, as it is the duration of these cycles that limit gains compared to those achieved by crop breeders. The high gains shown by methods of clonal breeding using rooted cuttings suggest that every effort should be made to solve propagation problems and implement these methods. In addition when initiating a programme with a new species, the limited gains that can be obtained *immediately* by mass selection, that is collection of open-pollinated seed from non-intensively selected trees should be exploited to the full whilst more sophisticated breeding methods are coming to fruition.

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*Appendix*

## CALCULATION OF GENETIC GAINS

**Example—Diameter**

The following data (provided by M. H. Bannister) were derived from pollinated families of *P. radiata* grown near Nelson.

$$\sigma_f^2 = \tfrac{1}{4}\,\sigma_A^2 = .1483 \therefore \sigma_A^2 = .5932$$

$$\sigma_t^2 = \sigma_w^2 + \tfrac{1}{4}\,\sigma_A^2 + \sigma_D^2$$

$$= 3.000$$

$$\sigma_p^2 = 0.0310$$

$$\sigma_I^2 = 3.1793;\ \sigma I = 1.783$$

Assume: $\sigma_D^2 = .5932$

$$\sigma_I^2 = 0$$

$$\sigma_C^2 = \tfrac{1}{4}\,\sigma_w^2 = .4905$$

heritability (narrow sense) = .187
heritability (broad sense) = .374
$\bar{x} = 10.17$ = population mean diameter.

Assume that all progeny and clonal tests involved 10 replications of 10 tree plots one location, and that $\sigma_{ge}^2$ (genotype x environment interaction) is zero.

**1. Collection of Open-Pollinated Seed from Selected Trees**

$$\Delta G = i\,\tfrac{1}{2}\,\frac{\sigma_A^2}{\sigma_I} \cdot \frac{1}{\bar{x}}$$

$$\text{e.g. } i = 2.06;\ \Delta G = \frac{2.06\,(0.5)\,.5932}{(1.783)(10.17)} = 3.4\%$$

**2. Seed Stand**

$$\Delta G = i\,\frac{\sigma_A^2}{\sigma_I} \cdot \frac{1}{\bar{x}}$$

$$\text{e.g. } i = 2.06;\ \Delta G = \frac{2.06\,(.5932)}{1.783\,(10.17)} = 6.7\%$$

NS

: derived from 29 open-

cations of 10 tree plots at
on) is zero.

### 3. Open-Pollinated Seedling Seed Orchard

*(a) Thinned progeny test*

$$\Delta G = \left( i_1 \tfrac{1}{2} \frac{\sigma_A^2}{\sigma_1} + i_2 \tfrac{1}{4} \frac{\sigma_{A'}^2}{\sigma_2} + i_3 \tfrac{3}{4} \frac{\sigma_{A''}^2}{\sigma_3} \right) \cdot \frac{1}{\bar{x}}$$

$$\sigma_A^2 = \sigma_{A''}^2 = .5932$$

$$\sigma_{A'}^2 = \sigma_A^2 (1 - \beta\nu')$$

$$\beta = \frac{\sigma_A^2}{\sigma_1^2} = .187 = \text{narrow sense heritability}$$

$\nu'$ from Finney (1957) = .9322

$$\therefore \sigma_{A'}^2 = .4898$$

$$\sigma_2^2 = \frac{\sigma_t^2}{100} + \frac{\sigma_p^2}{10} + \tfrac{1}{4}\sigma_{A'}^2$$

$$= .0300 + .0031 + .1225 = .1556$$

$$\sigma_2 = .3945$$

$$\sigma_3^2 = \sigma_t^2 + \sigma_p^2 = \sigma_w^2 + \tfrac{3}{4}\sigma_{A''}^2 + \sigma_D^2 + \sigma_p^2$$

$$= 3.000 + .0310$$

$$\sigma_3 = 1.741$$

Assume $i_1 = 3.37$; $i_2 = 1.27$; $i_3 = 1.40$

$$\Delta G = \left( \frac{3.37\,(0.5)\,.5932}{1.783} + \frac{1.27\,(.1225)}{.3945} + \frac{1.40\,(.4449)}{1.741} \right) \cdot \frac{1}{\bar{x}}$$

$$= (.5606 + .3944 + .3578) \cdot \frac{1}{10.17}$$

$$= 1.3124/10.17 = 12.9\%$$

*(b) Thinned bulked progenies*

$$\Delta G = \left( i_1 \frac{\tfrac{1}{2}\sigma_A^2}{\sigma_1} + i_4 \frac{\sigma_{A'''}^2}{\sigma_4} \right) \cdot \frac{1}{\bar{x}}$$

$$= \left( \frac{3.37\,(0.5)\,.5932}{1.783} + \frac{2.06\,(.5674)}{1.776} \right) \cdot \frac{1}{10.17}$$

$$= (.5602 + .6581) \cdot \frac{1}{10.17} = 1.2183/10.17 = 12.0\%$$

because $\sigma_{A'''}^2 = \tfrac{3}{4}\sigma_{A''}^2 + \tfrac{1}{4}\sigma_{A'}^2$

$$= .4449 + .1225$$

$$= .5674$$

$$\sigma_A^2 - \sigma_{A'''}^2 = .0258$$

$$\sigma_4^2 = \sigma_1^2 - .0258$$

$$= 3.1535$$

$$\sigma_4 = 1.776$$

4. Clonal Seed Orchard (without progeny test)

$$\Delta G = i \frac{\sigma_A^2}{\sigma_1} \cdot \frac{1}{\bar{x}}$$

e.g. $i = 2.67$

$$\Delta G = \frac{2.67\,(.5932)}{1.783\,(10.17)} = 8.7\%$$

5. Clonal Seed Orchard (with progeny testing)

*(a) 4 tester N.C. Design II control-pollinated test*

$$\Delta G = \left( i_1 \frac{\sigma_A^2}{\sigma_1} + 2\, i_2 \frac{\frac{1}{4}\sigma_{A'}^2}{\sigma_2} \right) \cdot \frac{1}{\bar{x}}$$

$$\sigma_2^2 = \frac{\sigma_t^2 - \frac{\sigma_{mf}^2}{4}}{100} + \frac{\sigma_p^2}{10} + \frac{\sigma_{A'}^2}{4} + \frac{\sigma_{mf}^2}{4}$$

Where $\sigma_{mf}^2 = \frac{1}{4}\sigma_D^2 = .5932/4 = .1483$)

$$\sigma_2^2 = \frac{3.00 - .0371}{100} + \frac{.0310}{10} + .1225 + .0371$$

$$= .1923$$

$$\sigma_2 = .4385$$

$i_2$ varies from 0.80 to 2.06. Assume $i_1 = 3.37$

$$\Delta G = \left( \frac{3.37\,(.5932)}{1.783} + \frac{.80\,(2)\,(.1225)}{.4385} \right) \cdot \frac{1}{\bar{x}}$$

$$= (1.1202 + .4470) \cdot \frac{1}{10.17} = 1.6170/10.17 = 15.9\%$$

*(b) Polycross or open-pollinated test*

$$\Delta G = \left( i_1 \frac{\sigma_A^2}{\sigma_1} + 2 i_2 \frac{\frac{1}{4}\sigma_{A'}^2}{\sigma_2} \right) \cdot \frac{1}{x}$$

$$\sigma_2^2 = \frac{\sigma_t^2}{100} + \frac{\sigma_p^2}{10} + \frac{\sigma_{A'}^2}{4}$$

$$= \frac{3.000}{100} + \frac{.0310}{10} + .1225$$

$$= .1556$$

$$\sigma_2 = .3945$$

e.g. $i_2 = 0.80$

$$\Delta G = \left( \frac{3.37\,(.5932)}{1.783} + \frac{.80\,(2)\,(.1225)}{.3945} \right) \cdot \frac{1}{10.17}$$

$$= (1.1202 + .4968) \cdot \frac{1}{10.17} = 1.6170/10.17 = 15.9\%$$

(c) *Clonal test for general combining ability*

$$\Delta G = \left( i_1 \frac{\sigma_A^2}{\sigma_1} + i_2 \frac{\sigma_{A'}^2}{\sigma_2} \right) \cdot \frac{1}{\bar{x}}$$

$$\sigma_w^2 = \sigma_t^2 - \tfrac{1}{4}\sigma_A^2 - \sigma_D^2$$

$$= 1.9619$$

$$\sigma_C^2 = 1.9619/4 = .4905$$

$$\sigma_2^2 = \frac{\sigma_w^2}{100} + \frac{\sigma_p^2}{10} + \left( \sigma_{A'}^2 + \sigma_{D'}^2 + \sigma_{I'}^2 \right) + \sigma_C^2$$

$$= \frac{1.9619}{100} + \frac{.0310}{10} + .4898 + .4898 + .4905$$

$$= 1.4928$$

$$\sigma_2 = 1.213$$

$$i = .80, \ \Delta G = \left( \frac{3.37\,(.5932)}{1.783} + \frac{.80\,(.4898)}{1.213} \right) \cdot \frac{1}{10.17}$$

$$= (1.1202 + .3230) \cdot \frac{1}{10.17} = 1.4432/10.17 = 14.2\%$$

6. Second Stage Clonal Seed Orchard

$$\Delta G = \left( i_1 \frac{\sigma_A^2}{\sigma_1} + i_2 \frac{\frac{1}{2}\sigma_{A'}^2}{\sigma_2} + i_3 \frac{\frac{1}{2}\sigma_{A''}^2}{\sigma_3} \right) \cdot \frac{1}{\bar{x}}$$

$$= \left( \frac{3.37\,(.5932)}{1.783} + \frac{2.06\,(.2450)}{.4228} + \frac{2.665\,(.2966)}{1.653} \right) \cdot \frac{1}{10.17}$$

$$= (1.1202 + 1.1937 + .4782) \cdot \frac{1}{10.17}$$

$$= 2.7921/10.17 = 27\%$$

7. Control-Pollinated Seedling Seed Orchard

(a) *Thinned progeny test*

$$\Delta G = \left( i_1 \frac{\sigma_A^2}{\sigma_1} + \frac{\frac{1}{2}\sigma_{A'}^2}{\sigma_2} + i_3 \frac{\frac{1}{2}\sigma_{A''}^2}{\sigma_3} \right) \cdot \frac{1}{\bar{x}}$$

$\sigma_2$ (as for open-pollinated orchard) = .3945

$\sigma_3$ (as for open-pollinated orchard) = 1.741

Assume: $i_1$ (at 1 in 1000) = 3.37

$i_2$ (at 1 in 4) = 1.27

$i_3$ (at 1 in 5) = 1.40

$$\Delta G = \left( \frac{3.37\,(.5932)}{1.783} + \frac{1.27\,(.1225)}{.3945} + \frac{1.40\,(.4449)}{1.741} \right) \cdot \frac{1}{10.17}$$

$$= (1.1202 + .3944 + .3578) \cdot \frac{1}{10.17} = 1.8724/10.17 = 18.4\%$$

(Continued overleaf)

(b) *Thinned bulked progenies*

$$\Delta G = \left( i_1 \frac{\sigma_A^2}{\sigma_1} + i_4 \frac{\sigma_{A'''}^2}{\sigma_4} \right) \cdot \frac{1}{\bar{x}}$$

As for open-pollinated orchard except initial phenotypic selection gain is doubled.

$$= \left( \frac{3.37\,(.5932)}{1.783} + \frac{2.06\,(.5674)}{1.776} \right) \cdot \frac{1}{10.17}$$

$$= (1.1202 + .6581)\,\frac{1}{10.17}$$

$$= 17.5\%$$

8. Two-Clone Orchard

$$\Delta G = \left( i_1 \frac{\sigma_A^2}{\sigma_1} + i_2 \frac{\frac{1}{2}\,\sigma_{A'}^2}{\sigma_2} + i_2 \frac{\frac{1}{4}\,\sigma_D^2}{\sigma_2} \right) \cdot \frac{1}{\bar{x}}$$

$$\sigma_m^2 = \sigma_w^2 + \tfrac{1}{2}\,\sigma_{A'}^2 + \tfrac{1}{4}\,\sigma_D^2$$

$$= 1.9619 + .2449 + .4449$$

$$= 2.6517$$

$$\sigma_2^2 = \frac{\sigma_m^2}{100} + \frac{\sigma_p^2}{10} + \frac{\sigma_{A'}^2}{2} + \frac{\sigma_D^2}{4}$$

$$= .0265 + .0031 + .2449 + .1483$$

$$= .4228$$

$$\sigma_2 = .6502$$

$$\Delta G = \left( 1.1202 + \frac{.80\,(.2449)}{.6502} + \frac{.80\,(.1483)}{.6502} \right) \cdot \frac{1}{10.17}$$

$$= (1.1202 + .3014 + .1825) \cdot \frac{1}{10.17} = 1.6041/10.17 = 15.8\%$$

9. Clonal Selection and Bulk Propagation

$$\Delta G = \bar{c} + \left( i_1 \frac{\sigma_G^2}{\sigma_1} \right) \cdot \frac{1}{\bar{x}}$$

where $\sigma_G^2 = \sigma_A^2 + \sigma_D^2 + \sigma_I^2$

$$= .5932 + .5932 + 0$$

$$= 1.1864$$

$$\Delta G = \bar{c} + \left( 3.37\,\frac{(1.1864)}{1.783} \right) \cdot \frac{1}{10.17}$$

$$= \bar{c} + 2.2404/10.17 = 22.0\% + \bar{c}$$

where $\bar{c}$ = average difference between seedlings and cuttings due to effect of propagation.

selection gain is doubled.

10.17 = 15.8%

lue to effect of propaga-

**10. Clonal Selection, Clonal Testing and Bulk Propagation**

$$\Delta G = \bar{c} + \left( i_1 \frac{\sigma_G^2}{\sigma_1} + i_2 \frac{\left(\sigma_{G'}^2 + \sigma_C^2\right)}{\sigma_2} \right) \cdot \frac{1}{\bar{x}}$$

$$\sigma_{G'}^2 = \sigma_G^2 (1 - \beta\nu')$$

$$\beta = \frac{\sigma_G^2}{\sigma_1^2} = .3732$$

$$\sigma_{G'}^2 = 1.1864 \, (1 - .373 \, (.9322))$$

$$= .7737$$

$$\sigma_C^2 = .4905$$

$$\sigma_2^2 = \frac{\sigma_w^2}{100} + \frac{\sigma_p^2}{10} + \sigma_{G'}^2 + \sigma_C^2$$

$$= \frac{1.9619}{100} + \frac{.0310}{10} + .7737 + .4905$$

$$= 1.2869$$

$$\sigma_2 = 1.134$$

$$\Delta G = \bar{c} + \left( 2.2404 + .80 \frac{(1.2642)}{1.134} \right) \cdot \frac{1}{10.17}$$

$$= \bar{c} + (2.2404 + .8918) \cdot \frac{1}{10.17} = 3.1322/10.17 = 30.8\%$$

**Variance Data for Bole Straightness**

The following data were derived from the same source as those on diameter:

$$\sigma_f^2 = \tfrac{1}{4} \sigma_A^2 = 3.8418 \therefore \sigma_A^2 = 15.37$$

$$\sigma_t^2 = \sigma_w^2 + \tfrac{3}{4} \sigma_A^2 + \sigma_D^2 = 20.262$$

$$\sigma_p^2 = 1.3880$$

$$\sigma_I^2 = 25.466$$

$$\sigma_1 = 5.047$$

Assume: $\sigma_D^2 = \sigma_I^2 = 0$; $\sigma_C^2 = \tfrac{1}{4} \sigma_w^2 = 2.1843$;

heritability (narrow sense) = heritability (broad sense) = .60;

$\bar{x}$ = population mean straightness

= 21.52

## *Appendix*

TABLE 1

(Table 1 from Nanson, A., Tables de la différentielle de selection dans la distribution normale (0, 1).).
Values of i (selection intensity) for different proportions of the population selected, P, for population sizes greater than 50.

| P | 0.00 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.0 | ∞ | 2.66 | 2.42 | 2.27 | 2.15 | 2.06 | 1.98 | 1.92 | 1.86 | 1.80 |
| 0.1 | 1.75 | 1.71 | 1.67 | 1.63 | 1.59 | 1.56 | 1.52 | 1.49 | 1.46 | 1.43 |
| 0.2 | 1.40 | 1.37 | 1.34 | 1.32 | 1.30 | 1.27 | 1.25 | 1.22 | 1.20 | 1.18 |
| 0.3 | 1.16 | 1.14 | 1.12 | 1.10 | 1.08 | 1.06 | 1.04 | 1.02 | 1.00 | 0.98 |
| 0.4 | 0.97 | 0.95 | 0.93 | 0.91 | 0.90 | 0.88 | 0.86 | 0.85 | 0.83 | 0.81 |
| 0.5 | 0.80 | 0.78 | 0.77 | 0.75 | 0.74 | 0.72 | 0.70 | 0.69 | 0.67 | 0.66 |
| 0.6 | 0.64 | 0.63 | 0.61 | 0.60 | 0.58 | 0.57 | 0.56 | 0.54 | 0.52 | 0.51 |
| 0.7 | 0.50 | 0.48 | 0.47 | 0.45 | 0.44 | 0.42 | 0.41 | 0.39 | 0.38 | 0.36 |
| 0.8 | 0.35 | 0.33 | 0.32 | 0.30 | 0.29 | 0.27 | 0.26 | 0.24 | 0.23 | 0.21 |
| 0.9 | 0.19 | 0.18 | 0.16 | 0.14 | 0.13 | 0.11 | 0.09 | 0.07 | 0.05 | 0.03 |

| | |
|---|---|
| 0.005 | 2.9 |
| 0.001 | 3.4 |
| 0.0005 | 3.6 |
| 0.0001 | 4.0 |
| 0.00001 | 4.5 |

## *Appendix*

### TABLE 2

Extract from Table 1 from Finney, D. J., The consequences of selection for a variate subject to errors of measurement. Revue Inst. Int. de Stat. 24: 1,3 1956.

| P Proportion of population selected | $\nu'$ | P Proportion of population selected | $\nu'$ |
|---|---|---|---|
| .95 | .1903 | .25 | .7583 |
| .90 | .2879 | .20 | .7813 |
| .85 | .3595 | .15 | .8051 |
| .80 | .4169 | .10 | .8308 |
| .75 | .4653 | .075 | .8453 |
| .70 | .5071 | .05 | .8619 |
| .65 | .5442 | .025 | .8833 |
| .60 | .5777 | .02 | .8888 |
| .55 | .6083 | .0125 | .8989 |
| .50 | .6366 | .01 | .9031 |
| .45 | .6631 | .005 | .9142 |
| .40 | .6881 | .001 | .9322 |
| .35 | .7121 | .0005 | .94 (approx.) |
| .30 | .7354 | .0001 | .95 (approx.) |

$$\sigma_{A'}^2 = \sigma_A^2(1 - \beta\nu')$$

where $\sigma_{A'}^2$ = reduced additive variance remaining in a group of selected plus trees.

$\sigma_A^2$ = additive variance in unselected (original) population.

$\beta = \frac{\sigma_A^2}{\sigma_p^2}$ = narrow sense heritability.

$\sigma_p^2$ = phenotypic variance.

$\nu'$ is tabulated above depending on the proportion of the population selected.

2.9
3.4
3.6
4.0
4.5

0.005
0.001
0.0005
0.0001
0.00001

# BREEDING POPULATIONS FOR RECURRENT SELECTION: CONFLICTS AND POSSIBLE SOLUTIONS*

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## ABSTRACT

Requirements for advanced generation breeding populations in forest trees are considered in terms of initial selection of parent genotypes, mating designs, and the nature of progeny plantings.

Large numbers of parent genotypes, 200 or more, are considered desirable, to minimise inbreeding, and to avoid loss of uncommon genes which might eventually prove valuable.

Mating designs should combine immediate efficiency of genetic gain with maintenance of effective population size. Several mating designs, commonly used to test parent genotypes or to estimate genetic parameters, are unsuitable for producing advanced generation breeding populations.

The most promising designs appear to be single-pair matings, and some modifications of the polycross. These are considered in detail, but further study is needed of the effects of non-additive genetic variance on their expected efficiencies. Final choice of mating design, however, may depend on availability of certain information from existing progeny trials, and on the possible need to fulfil other objectives, such as testing parental genotypes.

Progeny plantings could, in some circumstances, be designed entirely for efficient evaluation of individuals in relation to their family means. Clonal replication of seedlings should be explored as a procedure in selecting for traits with "all-or-none" expression.

## INTRODUCTION

Most existing tree breeding programmes were initiated long before any close analysis had been made of the problem of developing advanced generation breeding populations. However, the numbers of trees that are initially selected and involved in progeny tests strongly influence certain contributions to the overall selection differentials, and thus the genetic gains. They also control the effective size of the breeding population in the select generation of a recurrent selection programme, and the future degree of inbreeding. In fact the number of select parents, as well as the way in which these have been mated, may be such that the breeder has little option but to go back to the base population, greatly increase numbers of selections, and make a completely new series

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of matings to provide a proper base for recurrent selection. A case history from our own programme will illustrate this.

## NEW ZEALAND CASE HISTORY

In New Zealand there has been a gradual realisation of the implications of the requirements for effective rogueing of clonal seed orchards and for developing satisfactory populations for recurrent selection. When the *Pinus radiata* D. Don breeding programme was initiated in 1950, preliminary phenotypic selection of plus trees was highly intensive, averaging about one selected per 400 ha (1,000 acres) of plantation searched. The number of plus trees was further reduced by a second phenotypic screening to 14, and these clones were used in two small initial clonal seed orchards which were planted in 1958. In later extensions of these orchards this number was increased to 25 and then to 36, partly by retaining more trees from the preliminary field selection, and partly by additional field selection. It was subsequently appreciated that the extremely large numbers of trees screened in relation to those saved were giving selection differentials (the absolute differences between the means of populations under selection and means of select populations) that were little higher than could be achieved by screening far fewer trees. At the same time the small numbers of parents that were being selected would allow only very small selection differentials in a reselection of clones on the basis of progeny tests. Within existing orchards, any rogueing of worthwhile intensity would leave very few clones indeed; so these initial 14- to 36-clone orchards will be left as established.

It was therefore decided to make a further phenotypic selection in the field, greatly increasing the number of selections to about 800, and accepting a major reduction in degree of culling to about one tree per 1,000 (about one per 1.2 ha (3 acres)). Provision could thus be made for more intensive reselection of the clones for immediate seed production on the basis of progeny test performance, i.e., from 800 down to about 30. In the long-term breeding population, however, it is anticipated that as many as 200 of these genotypes may be represented as parents.

The large number of selections in turn raised practical problems of evaluating progenies effectively, quickly, and economically. It is convenient with the serotinous cones of *P. radiata* to collect open-pollinated seed from the original parents, and this is now used to establish progeny tests as soon as possible after selection. Since it is unlikely that there is much local differentiation among the base population that was screened, we are assuming that satisfactory rankings of genotypes for general combining ability, or GCA (at least with respect to the base population), will be obtained from the tests. An additional 80ha (200 acres) of seed orchard are now needed in order to meet seed requirements, and these new orchards will be established with the intention of doing a 2/3 to 3/4 rogueing by clones on the basis of results from the open-pollinated tests. In addition, 8 to 10 years hence the first of the clonal orchards will come due for re-establishment as it will be getting too tall for economic seed collection. These orchards will then be progressively replanted with ramets of top-ranked clones as determined by the open-pollinated tests. Quite intensive reselection will be possible if the 800 clones are to be reduced to as few as 20 to 40.

These orchards of the best progeny-tested genotypes should in fact take care of improved seed production to the year 2000 and beyond. Having achieved an acceptable strategy which will take us to this point, the requirements for the second generation selection and the derived orchards came under consideration.

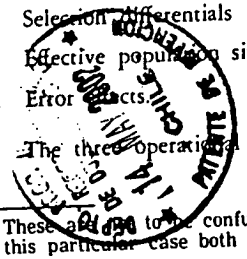
None of the existing progeny tests is really suitable as a new population for selection. Wind-pollinated progenies* collected from within the first two series of orchards with 14-25 clones will only allow limited selection on the basis of progeny (family) means, if an adequate effective population size is to be maintained, and the best individuals within progenies may well be sired by a restricted number of pollen parents. Selecting within bulked seed collections from these orchards is quite unsuitable because it could result in an even smaller number of effective parents. The few control-pollinated progenies that are available are from a very restricted number of parent trees. The extensive wind-pollinated progeny tests from the recent selections have the major disadvantage of being sired by completely unselected parents. Once the new orchards of much larger numbers of clones become productive it would be possible to collect seed by clones and plant these offspring for future selection. The main disadvantage in this approach stems from the variation in the time that different clones take to start producing abundant pollen, since seed collection must be delayed if there is to be any chance of obtaining a reasonably balanced representation of pollen parents. It thus appears that some form of control-pollinated mating programme will be necessary to produce second-generation material.

## GENERALISED REQUIREMENTS FOR A BREEDING POPULATION FOR RECURRENT† SELECTION

For a given base population the parameters which directly influence long-term genetic gain are:

1. Efficiency of estimating the genotypic values of trees in the second and subsequent generations, thence in identifying the superior individuals.
2. Selection differential between the base population and phenotypic selections of first generation.
3. Selection differentials in the second generation and subsequent generations.
4. Effective population size and amount of inbreeding.
5. Error effects.

The three operational determinants of a breeding population, namely number of



* These are not to be confused with the wind-pollinated progenies discussed elsewhere; in this particular case both seed and pollen parents are select.

† For detailed discussion on tree breeding methods, of strategies, and terms used, see Shelbourne (1969).



parental genotypes, the mating design, and the progeny planting will be considered later, largely in the light of their influence on the above parameters.

### *Identification of Desired Genotypes*

The estimates of the genotypic value of an individual can be improved by using information concerning its relatives as well as itself. Most commonly the relatives include its progeny (half-sibs and full-sibs) and its own sibs (half and full), but they can also include the parent ortets*, ramets from the parents, and of course ramets from the individual itself. The information can be combined by means of a selection index (not to be confused with the multiple-trait index) which will depend on the heritability, the degree of relatedness with the individual, and the number of relatives within each class. The wider the range of relatives for which information is available the better the estimate of the genotypic value of the individual (Osborne, 1957; Namkoong, 1966). We, however, must be concerned with whether the features of mating designs and progeny plantings which provide additional information are worth the cost in terms of the effort involved or its effect on selection differential and effective population size. This must be considered in relation to the biological constraints involved in tree breeding (cf. Libby, 1969). Two circumstances which contrast with the situation in animal breeding, for instance, are the monoecy of parents (i.e., the ability to produce both pollen and seed) and the feasibility of obtaining half-sib progenies from a single cross.

All further discussion is based on the assumption that selection is to be made on the basis of combined information for both the individuals in question and their relatives.

### *Selection Differentials*

Genetic gain from selection is normally directly proportional to the selection differential for that stage of selection, which in turn is controlled by the proportion of the selection units, be they trees or families, that is saved. It follows, then, that for a given investment in the programme, selection differentials must be maximised in order to maximise genetic gain. However, in an advanced generation breeding population there are various conflicts in requirements. Selection differentials will normally be increased at the expense of effective population size, at least for a finite resources model. When a selection index is being used to combine information concerning both the individual and its relatives, the concept of the selection differential becomes less simple; we can no longer talk of between-family and within-family selection differentials. Nevertheless, we can say that when the number of families is increased, family differences will make a greater contribution to the overall selection differential. However, under a finite resources model the increase in number of families would decrease the contribution made by the differences between individuals within families to this differential. The net effect on selection differential of the allocation of a finite number of individuals between and within families is notably small over a wide range of alternative family sizes and thus the numbers of families possible.

* Ortet = original plant from which the ramets of a clone are derived.

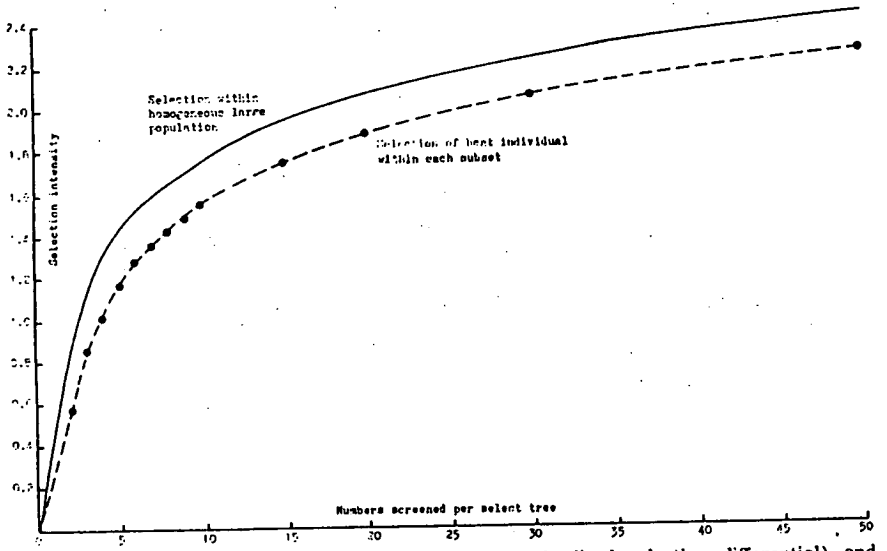



FIG. 1—Relationship between selection intensity (standardised selection differential) and number of trees screened per select tree.

From Fig. 1 it can be seen that the selection differential increases curvilinearly with the number of individuals screened for each one selected. Thus the increase in selection differential drops off rapidly in relation to additional effort with increasing number of trees screened for each one selected. The lower curve (dashed line) shows that when the best individuals are selected within finite groups or subsets the selection differentials are lower than when the same proportion of individuals is saved from a homogenous infinite population. The relative drop in selection differential becomes greater as the groups become smaller.

The general nature of the relationship would argue, when it is desired to maintain effective population size and when it is impossible to rank the parents effectively prior to crossing, for a spreading of contributions to the overall selection differential between progenies and within progenies, and between seed parents and pollen parents among progenies. In practice, however, this will often be achieved only with an excessive effort in relation to the number of parent genotypes involved.

These few considerations illustrate the point that selection differentials at different levels are subject to numerous countervailing effects, so that comparisons between certain breeding strategies can only be made on the basis of detailed calculations of gain expectations for varying proportions of the population selected and for traits with varying heritabilities.

### *Effective Population Size*

Our contention is that the effective size of the breeding population should be large. Provided large numbers of first generation selections are mated to produce large numbers of families and of individuals within families, selection differentials can be kept



high, and yet the frequency of related matings kept low and inbreeding minimised. We do not insist on strict preclusion of mating among related individuals but rather that the probability of related matings be kept low, if not negligible, over successive generations; many mating designs which with finite populations ensure complete outcrossing in early generations may accentuate inbreeding later on.

A large effective population size should also insure the breeder against having to meet new criteria for selection when the programme is already under way, such as having to breed for resistance to a freshly introduced pathogen. This reasoning is intuitively attractive and in general terms has been advanced in the past, e.g., by Zobel and McElwee (1964), with the concept of the tree bank.

However, some recent theoretical work by Libby (1969b) indicates that, starting from only a few parent genotypes, it is possible by correspondingly increasing family sizes, to achieve overall selection differentials which give large if not almost maximal expectation of genetic gain. But there is a catch in the classical genetic models*: they have been formulated on the simplifying assumption that there are only two possible alleles, or alternative genes, per locus, each present at virtually equal frequencies within the population. We must insure against the possibility of losing valuable genes which are present in the base populations at low frequencies. To be sure of recovering such genes when and if required, it would be necessary to maintain a large breeding population.

Total preclusion of mating among relatives may involve costly and inconvenient constraints on the mating design, and yet there is no certainty that the theoretical occurrence of inbreeding will eventuate. There is reason to believe that when pollination is made by a mixture of related and unrelated pollen, preferential outcrossing will occur even when self-fertility is quite high (Barnes *et al.*, 1962; Sarvas, 1962).

### *Error Effects*

These include items of variance which are confounded with the genetic variance that can be utilised in the breeding programme. Their presence will naturally reduce genetic gain. The classic case is special environmental variance among individual seedlings within families, but specific combining ability (SCA) if present will be an error item both among full-sib families and among individuals within families.

## NUMBERS OF PARENTS SELECTED

The desirability of initially selecting a large number of clones has already been discussed, but even so the precise number is still very arbitrary. However, we would think in terms of 200 or more, and certainly not the 20-30 which have formed the base of some programmes.

## MATING DESIGNS

### *Objectives*

Most mating designs in forest tree breeding are used for one or more of several possible objectives:

1. *Screening or ranking* of genotypes and families, genotypes for GCA, families for

* This has occurred to Dr W. J. Libby (pers. comm.) independently.

mean GCA and in certain situations for the SCA of their parents when crossed together.

2. *Estimation of components of variation,* which include additive genetic variance (which determines GCA), non-additive genetic variance (which determines SCA), environmental variance and genotype-environment interactions, as well as phenotypic and genotypic correlations between traits. From these statistics can be calculated heritabilities, optimum weighting of different characters, and expectations of genetic gain.
3. *Producing the population for the second generation of selection* from the offspring of initially selected parents.
4. *Direct estimation of realised genetic gains,* for purposes of evaluating the progress to date.

Thus, when we are considering the merits of a mating design for producing the base for an advanced generation of selection we should take some account of the need for fulfilling other objectives. We will consider several current mating designs, and then two designs or groups of designs which do not appear to be in common use.

Before going any further, however, we must state that our immediate concern will be entirely with utilising GCA. Screening for SCA, except perhaps at the level of interpopulation crosses, is laborious, and can only be done satisfactorily in experiments designed essentially for that purpose. Even if high SCA is recognised for several combinations of clones its advantages have to be weighed against the costs of a method of propagation which will recover this form of genetic gain. For present purposes, therefore, SCA is of concern mainly as a potential source of bias in screening for GCA.

### *Currently Used Mating Designs*

WIND-POLLINATED PROGENIES, if they approximate closely to half-sib families, provide a quick and economical method of estimating GCAs of individual genotypes and GCA variance among the sample of parents from which the seed was collected. The estimates of GCAs of the selected clones will, of course, only apply with respect to the base population and not necessarily with respect to a selected population of clones. The GCAs (and to a lesser extent GCA variance) with respect to the select population could, in certain situations, be substantially different, but in the balance we consider this possibility unlikely. It is also possible, under certain circumstances, to predict genetic gains from a clonal seed orchard from tests of such progenies. The progenies, however, are not attractive as a base for second-generation selection because the pollen parents are normally unselected, and the half-sib relationship is not as efficient as higher levels (e.g., full-sib).

POLYCROSS mating designs normally utilise a standard pollen mix from a number of parents, which is mated to all parents under test. This can be expected to provide good estimates of GCAs and GCA variance, particularly if the pollen is from the genotypes under test, with only one cross per clone being needed. These designs can also provide good estimates of the genetic gains which will be achieved with a particular seed orchard. However, a simple polycross with a limited number of parents cannot be recommended as a base for second generation selection; most of the trees selected within each progeny are likely to come from the same few pollen parents, which would seriously reduce the effective population size. Again, the half-sib level of relationship is less efficient than higher levels of relationship in index selection.



NORTH CAROLINA (NC) DESIGN II is an example of a *Factorial Design**. It usually involves about four genotypes used as males (or females) each mated to all of the remaining genotypes. GCA of each female is estimated from the half-sib family average of four full-sib families (with the four testers). In relation to the parent genotypes under test the design allows estimates of GCA and SCA variances. As in all factorial designs each parent genotype is involved in full-sib matings with several others. The successful completion of all the specified crosses is difficult and time-consuming, and yet missing crosses involve the loss of considerable information. For purposes of selecting among offspring, efficiencies due to the availability of both full-sib and half-sib information are normally vitiated by one or more of three effects: (1) reduction in the number of genotypes that can be used as parents in the mating design, (2) reduction in the number of individuals per family, or (3) increase in the size and cost of the experiment.

In connection with (1) the effective population size under the NC II design is much reduced by the fact that four parents contribute at least half the germ plasm of the progenies, and it will be further reduced if any discrimination is to be made against the progenies of the poorer testers. Unless phenotypic selection is highly reliable or there is prior confirmation of the GCAs of the testers there is always the risk that through random sampling error the testers could have been appreciably poorer than the average for the phenotypically selected clones. Even where several NC II designs are pooled they still involve all the operational inefficiencies which are inherent in the unequal contributions made by the parents, unless the tester parents have been proven to be outstanding.

The NC DESIGN I is an example of an *Hierarchical Design,* involving the mating of different groups of females (common sex) to each male (rare sex), or vice versa. It is normally used for the estimation of GCA and SCA variance components, but is not satisfactory for estimating GCA for individual genotypes, and selection on the basis of sib-family performance will be biased by SCA effects, unlike the polycross. As with the NC II the preponderance of germ plasm contributed by the limited number of trees of the rare sex reduces effective population size, particularly if discrimination is to be made against the poorer rare-sex parents. The use of parents which are known to be the best clones as the rare sex will give the best overall selection differential, and thence a high gain expectation. However, it is only for these rare-sex parents that the design affords estimates of GCA which are not fully confounded with SCA, and with these parents already proven such estimates of GCA would be superfluous. In the context of forest tree breeding it is not always possible to rank the select trees on phenotypic performance, for example, when the best trees are selected

* A factorial design involves the mating of each of **a** parents to each of **b** parents to give (**a** × **b**) crosses. A select population can be subdivided into a number of such cells.

from very different sites. Even if ranking is possible, biological constraints, such as the "best" trees producing only enough pollen to be used as common sex parents, are likely to reduce actual gains.

The DIALLEL CROSS is the most elaborate full-sib mating design. In its complete form $n$ parents are crossed in all possible combinations, which include selfings and reciprocal crosses, to give $n^2$ crosses. When the selfings and reciprocals are omitted the design is known as the half-diallel. It provides the most comprehensive genetic information for the material employed, since it furnishes both half-sib and full-sib information concerning progenies of all the parent clones. It is possible to select on the basis of GCA information for both seed- and pollen-parents, but unless the mating design involves a very large number of crosses the number of parents that can be used will not allow a large effective population size.

The large number of crosses and thus the work load involved in relation to the information available on GCA is such that the diallel cannot be recommended as a means of establishing an advanced breeding population in which further gains are sought on the basis of recovering GCA. The work load increases roughly according to the square of the number of parents used, which rules this design out when we want to ensure a large effective population size.

Partial and disconnected diallels permit the employment of larger numbers of parent clones, but in common with factorial mating designs, they are inherently inefficient by depending on several full-sib crosses in order to obtain half-sib information (which may be very imprecise) for each parent.

In practice the diallel cross and its modifications are mainly useful in providing efficient estimates of SCA variance and in screening for SCA among crosses. Improved estimates of GCA variance can be obtained by using partial diallels and disconnected diallels, which allow larger numbers of parents to be used, but this will be at the price of inferior GCA estimates for individual clones. Missing crosses (families), however, have a serious effect on the recovery of information.

### *Disconnected Single-Pair Mating Design and Modifications of the Polycross*

We will now consider some mating designs which do not appear to have been in regular use, but which we feel have the greatest promise for the current purpose. Firstly there is the SINGLE-PAIR mating design, proposed by Libby (1968, 1969b), which involves the mating of disconnected pairs of genotypes, so that each genotype is only mated to one other in the entire series. Then there are three alternative forms of the NESTED POLYCROSS, and finally the TESTER POLYCROSS.

The advantages and disadvantages of the single-pair mating design compared with the simple polycross will be reviewed, with some reference to other mating designs. The problem of comparative expectations of genetic gain for these two designs when non-additive gene effects are present will then be discussed. Lastly we will consider how the modifications of the polycross may mitigate certain disadvantages of the simple polycross.



Table 1 lists the main advantages and disadvantages of single-pair mating when compared with the polycross.

Under an additive genetic model at least, the expected efficiency of single-pair mating in identifying the best genotypes is maximum for most, and near maximal for all heritabilities and population structures, compared with other mating designs. This is in spite of the risk of wasting outstanding parents through chance mating with inferior ones. Unlike single-pair mating, the polycross is subject to the constraint of each parent receiving pollen in which the mean breeding value is that of the select population in general, and the family information which the polycross affords is inferior. The operational simplicity of single-pair mating is very attractive, although the polycross still compares well in this respect with some other designs. Another attractive feature of single-pair mating is the efficiency with which it maintains effective population size. However, our proposals for modifications of the polycross should partially offset its disadvantage in this respect. The inability of single-pair mating to provide estimates of GCA is an important disadvantage, the implications of which will be considered later.

TABLE 1—Advantages and disadvantages of the single-pair mating design compared with the polycross

| Single-Pair Mating | Polycross |
|---|---|
| **Advantages** | **Disadvantages** |
| 1. High expectation of genetic gain, under additive genetic model | Somewhat lower expectation of genetic gain |
| 2. Maintains effective population size with maximum efficiency | Risk of appreciable loss of effective population size |
| 3. Only one cross per two parents | One cross per parent |
| 4. Direction of crosses immaterial—only seed or only pollen needed from any one parent | Seed and pollen needed from each parent |
| 5. Pedigreed breeding method—both parents identified | Pollen parent not identified |
| **Disadvantages** | **Advantages** |
| 1. No estimates of GCA available for individual parents | Estimates of GCA available for all parents |
| 2. Bias in selection on basis of family means due to non-additive gene effects | Very little corresponding bias due to non-additive gene effects |
| 3. Risk of wasting outstanding parents through chance mating with inferior ones | Assured utilisation of outstanding parents |

The net effects of SCA or non-additive gene effects on the comparative expectations of genetic gain have not been explored, but we will review the exepcted genetic

parameters for single-pair mating and polycross families respectively (c.f. Kempthorne, 1959, p. 423), in order to illustrate the nature of the problem.

Firstly, consider the expected variance *between* family means. For single-pair mating the expectation (assuming random mating) is:

$$\tfrac{1}{2}\,\sigma^2_A + K_{s\text{-}p}\,\sigma^2_{NA} + m\,\sigma^2_{W(s\text{-}p)}$$

where $\sigma^2_A$ = additive genetic variance

$\sigma^2_{NA}$ = non-additive genetic variance

$K_{s\text{-}p}$ = at most $\frac{1}{4}$, and can be appreciably less (s-p = single-pair)

$\sigma^2_{W(s\text{-}p)}$ = variance within full-sib families = error

m = reciprocal of the number of individuals per family or a somewhat greater value, depending on the field design

Alternatively this expectation can be written,

$$2\,\sigma^2_{GCA} + \sigma^2_{SCA} + m.\ \text{Error}$$

For polycross families (assuming a half-sib model) the corresponding expectation of variance *between* family means is:

$$\tfrac{1}{4}\,\sigma^2_A + K_p\sigma^2_{NA} + m\,\sigma^2_{W(p)}$$

where $K_p$ = at most 1/16, and can be considerably less (p = polycross)

$\sigma^2_{W(p)}$ = variance within half-sib families = error

Alternatively this expectation can be written,

$$\sigma^2_{GCA}{}^{*} + m.\ \text{Error}$$

For any design, when the crosses are made between select parents, the $\sigma^2_A$ component of between-family variance will be reduced to $\sigma^2_{A'}$, according to the relationship,

$$\sigma^2_{A'} = \sigma^2_A\,(1 - \beta\,v')$$

where $\beta = (\sigma^2_A)/\sigma^2_P$ = (narrow-sense) heritability

$\sigma^2_P$ = phenotypic variance

and $v'$ is a parameter depending on the proportion of the population saved (Finney, 1956). For example, for heritability = 0.6, proportion saved = 1 : 1,000, $\sigma^2_{A'}$ = 44% of $\sigma^2_A$

It can be seen that the differences between families contain twice as much additive genetic variance with the single-pair mating design as with the polycross. The larger fraction of the total additive genetic variance makes for a more efficient contribution of family information towards estimating the genotypic value of an individual. However, the estimates of mean parental GCA are biased by SCA in the single-pair mating design, unlike the polycross. The family information, therefore will be contaminated by a much smaller fraction of the non-additive genetic variation with the latter design. This bias will be intensified when select parents are used, because the reduction of $\sigma^2_A$ to $\sigma^2_{A'}$ will cause a relative increase in the non-additive components. The difference between $\sigma^2_A$ and $\sigma^2_{A'}$ is minimal when heritability is low,

* It must be noted that GCA is not entirely equivalent to additive gene effects, since it is contaminated with small proportions of certain non-additive variance components. In theory at least, prediction of response to selection by means of GCA estimates is therefore subject to slight bias.



but under such conditions an appreciable proportion of the genetic variance may be non-additive. This sort of situation is a real possibility when we are considering a highly composite trait such as overall desirability. When heritability is high non-additive genetic variance would be small in relation to the additive component, but it would show a greater relative increase on account of the greater difference between $\sigma^2_A$ and $\sigma^2_{A'}$.

We will now consider variance *within* progenies. For single-pair mating the expectation is:

$$\tfrac{1}{2}\,\sigma^2_A + K\,\sigma^2_{NA} + n\,\sigma^2_E\ (=\ \sigma^2_{W(s\text{-}p)})$$

where K = at least $\frac{1}{4}$, but less than 1.

$\sigma^2_E$ = environmental variance

n = reciprocal of the number of ramets per seedling clone (where cloning of seedlings within families is carried out), or a somewhat greater value, depending on the experimental design

For polycrossing the corresponding expectation of variance *within* families is:

$$\tfrac{1}{4}\,\sigma^2_A + K\,\sigma^2_{NA} + n\,\sigma^2_E\ (=\ \sigma^2_{W(p)})$$

where K = at least 15/16, but less than 1.

In the case of polycrossing with select parents $\frac{1}{4}\,\sigma^2_A$ should be rewritten,

$$\tfrac{1}{2}\,\sigma^2_A + \tfrac{1}{4}\,\sigma^2_{A'}$$

where the first term represents recombinational variance, which is assumed to remain unchanged by selection, and the second term represents variance deriving from the different pollen parents.

Within families the position concerning expectations of variance is largely reversed compared with that between families. There is more additive genetic variance within polycross families than within single-pair families, $\frac{1}{4}\sigma^2_A$, compared with $\frac{1}{2}\sigma^2_A$. Against this, the estimates of genotypic values of individuals are biased by more non-additive genetic variance within polycross families than within single-pair families, roughly $\sigma^2_{NA}$ compared with $\frac{1}{4}\sigma^2_{NA}$. Nevertheless, within polycross families the non-additive genetic variance is less in relation to the additive genetic variance present than within single-pair families, except when the additive genetic variance between parents has been much reduced by initial selection.

To conclude the consideration of non-additive gene effects, it is far from clear, without extensive theoretical work, what the net results of such effects are on the comparative expectations of gain from polycross and single-pair designs. Greatest efficiency in achieving genetic gain will only be obtained with an appropriate between- and within-family selection index, but we must seek a strategy for which the expected efficiency is robust not only with respect to deviations from the assumed heritability, but also with respect to deviations from a purely additive genetic model.

Let us now consider possible modifications of the polycross. In the NESTED POLYCROSS, the select parents are grouped into a series of nests, perhaps 20-30 clones each. The nesting should help maintain large effective population size because it should reduce the possible preponderance of the contribution of occasional really outstanding pollen parents to the selected offspring. In any case the selections from different nests should be completely unrelated. Subsequent crosses between nests should

therefore involve no inbreeding at all. If the nests are of reasonable size the sampling error variance between nests should be small and the assumption that nests are all of equivalent average genotypic value should be reasonably reliable. Conversely, very small nests will be subject to appreciable genetic sampling error, so that selection within them on the basis of family means will tend to be inefficient. The nest is a highly convenient operational subdivision of the programme, and a series of nests could be crossed and planted out one after another over a period of years. Minor imperfections in the mating design should be acceptable; it would not be important, for instance, if pollen was unavailable or only in short supply from one or two parents in a nest.

There are three types of polycross nest to consider; the complete nest, the incomplete nest, and the overlapping nest. Within the COMPLETE POLYCROSS NEST all parents are used both as seed parents and in the pollen mix. This allows estimation of GCA for all parents. Theoretically, some self-fertilisation could occur, but in practice this may be negligible. Of the selfings that do occur, very few if any would be included in the selected offspring. This probable condition of ineffective self-fertilisation will tend to maintain effective population size and reduce inbreeding. In the OVERLAPPING POLYCROSS NEST design the parents from one nest are pollinated by the mix from another nest, while the parents from the latter nest would be pollinated by the mix from a third nest, and so on. Self-fertilisation is completely precluded, but this would appear to be of no real advantage, reducing the assurance of outcrossing at a later date.

INCOMPLETE POLYCROSS NESTS involve the use of part, say half, or the genotypes within each nest as seed parents, and the remainder for the pollen mix. This will reduce the number of crosses required. Against this, estimates of GCA will not be obtained for the pollen parents, and it will be impossible to select their offspring deliberately.

Similar in concept to the incomplete polycross nest is the TESTER POLYCROSS, which may or may not be subdivided into nests, according to operational convenience. Here the pollen mix is derived from parents all of proven GCA, and it is mated with newly selected or "candidate" trees. These new selections will all be screened for GCA with respect to the main breeding population and at the same time they will have been mated to the best available parents. The proven GCA of all pollen parents should ensure that they are represented in a satisfactory balance among the selected offspring. The use of parents from a well-established clonal archive should avoid all problems with availability of pollen. After the second generation is reached the tester polycross can be made at any time, so that later generations need no longer be discrete.

On the basis of these considerations it is clear that a serious investigation should be made of expected genetic gains and effective population sizes under these types of polycross.

## PROGENY PLANTINGS

In planting out a breeding population from a completed mating programme in a field design, and therefore a given number of families, we are faced with several objectives. These may be listed as:



1. Estimating mean value of each family, which automatically furnishes genetic information concerning parents.
2. Providing satisfactory within-family contributions to selection differentials.
3. Providing genetic information concerning the individual trees with respect to their family means.

As stated earlier the between-family and within-family information can be combined by means of an index in order to make the actual selection.

The first two of the three objectives are both achieved by planting a large number of individual genotypes from each progeny. However, an increase in selection differential will be vitiated if environmental variance is increased at the same time, because the phenotypic value of the individual will become a less reliable guide to its genotypic value. It is axiomatic, therefore, that within the framework of a satisfactory experimental design, tree-to-tree environmental variation be minimised by good establishment techniques.

Maximum genetic information for individuals within progenies can be achieved by clonal replication of seedlings (cf. Libby, 1964, 1969a, 1969b), and the relative gain in efficiency will be greatest when heritabilities are low. However, for a given size of experiment this can only be done by reducing both the within-family contribution to the overall selection differential, and to a lesser extent, the precision of estimating family means. Libby (1969b) found that these latter factors offset the greater precision in identifying good genotypes, and clonal replication could not be justified in comparison with selecting less efficiently among more genotypes. However, the models did not incorporate the case of significant genotype-site interactions, a factor which would favour the use of seedling clones.

The previous theoretical work has been based on the assumption that the conceptual character under selection is a continuously varying metric trait. This is often not so. Malformation, which is a major problem in our tree crops, commonly shows an "all-or-nothing" expression; presumably it is partly governed by a threshold effect and partly by factors of chance. Replication of seedling clones may be essential to reasonably effective selection of individual genotypes for freedom from malformation. Much, of course, depends on the feasibility of cheap and rapid replication of seedling ortets, but this possibility does warrant further study.

It is evident that where seedlings can be replicated clonally there may be conflicts between obtaining progeny information on one hand, and individual genotype information on the other. In extreme cases there may be situations in which the two objectives can best be achieved with separate field plantings from the same mating design. Sometimes, however, such as when wind-pollinated progenies approximate to half-sib families we can already obtain a separate progeny test which will provide genetic information concerning parents and any resulting progenies. The overriding concern will then be to obtain information concerning individuals within progenies. To obtain more precise information at this level each family could be a self-contained trial, without further regard to comparisons among families. Although self-contained, these single-family trials could each embrace several sites.

## TOWARDS A STRATEGY

Although the expectations of genetic gain require much further investigation, the final choice of a mating design and a progeny test design for producing an advanced generation breeding population may hinge on the suitability and availability of wind-pollinated progenies for estimating parental GCAs. We are basing our case on the proposition that in any event, estimates should be obtained of GCA for all phenotypically selected parents. Let us consider the reasons for this assumption.

If it is desired to reselect among the initial selection of clones in order to rogue or re-establish first-generation clonal seed orchards, then the need for estimates of GCA for clones is self-evident, and the sooner they are obtained the better. This consideration aside, a knowledge of GCAs provides insurance in case a change in criteria for selection is required. If in this event we have to utilise genes which are present at low frequencies within the base population, we cannot be assured of a normal distribution of breeding values among the initially selected parents for the newly desired traits. This, in turn, would mean that existing mating combinations could be much less efficient than conventional models suggest they are in utilising those genotypes which are valuable under the new circumstances. A selective remating of parent genotypes in new combinations would then be highly desirable. At the same time it would be desirable to introduce freshly selected clones from the base population in order to maintain the effective size of the breeding population, and it would be necessary to be able to identify and use the best genotypes within the initial selection to mate with the newcomers. The availability of GCA estimates for all the original select genotypes will enable the best to be identified. Those genotypes of highest GCA could be used in a pollen mix to mate with the new selections; this is an example of a tester polycross and it should be highly efficient. Furthermore, this provision for efficient incorporation of new selections into the breeding population should be made regardless of any major changes in selection criteria.

If wind-pollinated progenies do give good estimates of parent GCAs *and are already established in a trial,* then the single-pair mating design would probably be preferred on grounds of its operational convenience and the efficiency with which it can maintain effective population size. The bias caused by SCA in the between-families component of selection can be eliminated by the external availability of GCA estimates. As indicated earlier, the field design in which the pair-crosses are planted need only be concerned with selection within families. If reliable progeny test information can be obtained within a few years, it would be possible to rogue the clones before control-crossing is done. However, our dependence on the assumption of a half-sib model does highlight a need for a good knowledge of pollination behaviour in the field. If a wind-pollinated progeny trial has not yet been established, and is not required for providing early information whereby a clonal seed orchard may be efficiently rogued or re-established, the case is not so clear-cut. The cost of planting two field trials must then be weighed against the advantage of being able to specialise the two functions of evaluating families and individuals within families respectively.

Where wind-pollinated progenies are difficult to obtain and/or do not give reliable estimates of GCAs we would look to a mating design which will achieve this objective



and at the same time be nearly optimal for advanced generation selection. In this case some modification of the polycross could be very suitable. It is possible, though, that separate plantings might be desirable to meet these different objectives.

Figs. 2 to 5 illustrate alternative flow charts, depending on the reliability of wind-pollinated progeny tests and on how quickly they provide information. All charts are

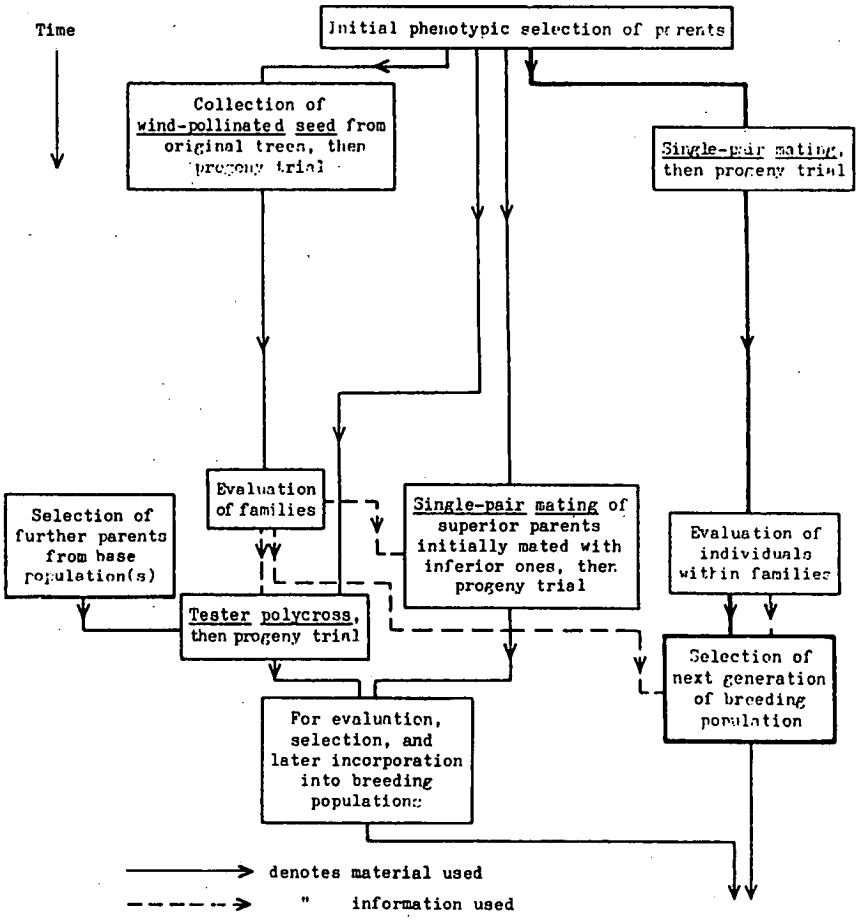



FIG. 2—Flow Chart 1. Suggested deployment of material in mating designs and use of information to produce advanced generation breeding populations

Conditions: (1) Wind-pollinated progenies equivalent to half-sib families.

(2) Early estimates of GCA not available from the wind-pollinated progeny trial.

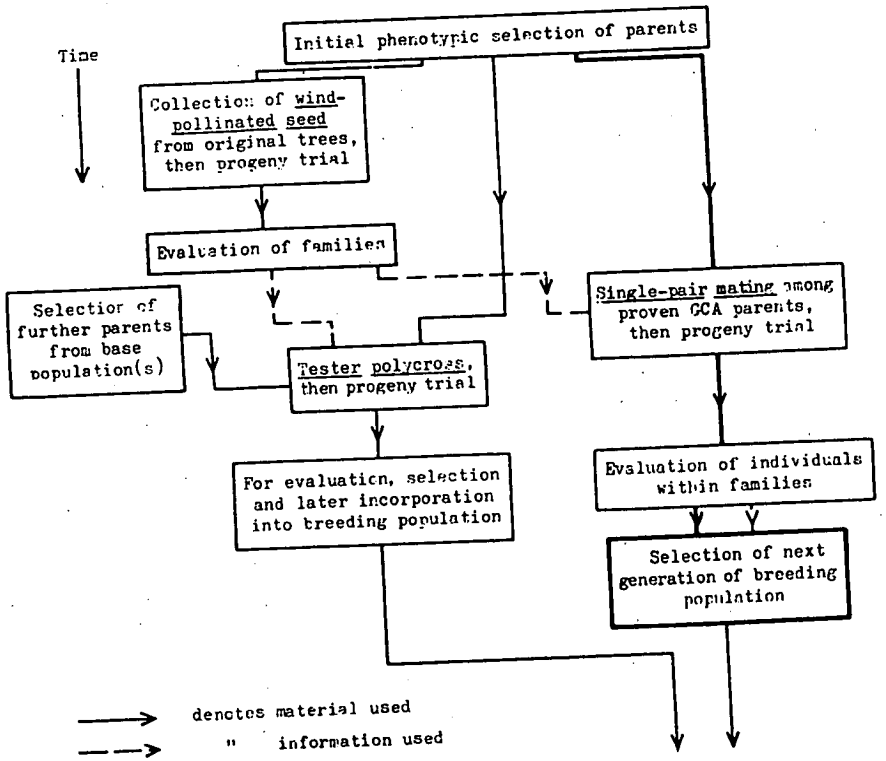



FIG. 3—Flow Chart 2. Suggested deployment of material in mating designs and use of information to produce advanced generation breeding populations

Conditions: (1) Wind-pollinated progenies equivalent to half-sib families.
(2) Early estimates of GCA available from progeny trial.



drawn to the same arbitrary time scale. For simplicity of argument it is assumed that rarely selected genotypes cannot contribute worthwhile genetic information. Fig. 2 covers the case of wind-pollinated progeny trials providing reliable information, but only with a considerable delay. For Fig. 3 the situation differs in that the relevant information can be obtained with little delay. Fig. 4 applies where wind-pollinated progenies cannot provide reliable information and where any progeny trial can only be evaluated after considerable delay. For Fig. 5 the situation differs from that in Fig. 4 in that progeny-test evaluations can be made with only little delay. Here the second generation can be produced by two main pathways, the relative importance of which may be determined by the particular circumstances.

In all cases provision is made for the introduction of fresh selections of clones, which can contribute to the breeding population perhaps after the second generation of



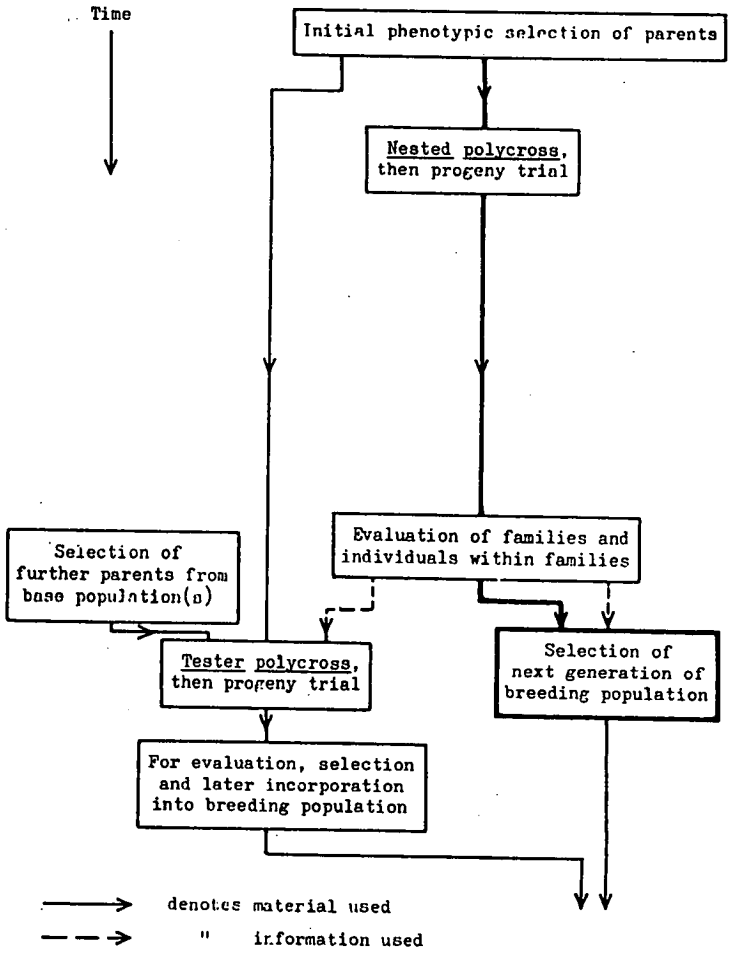



FIG. 4—Flow Chart 3. Suggested deployment of material in mating designs and use of information to produce advanced generation breeding populations

Conditions: (1) Wind-pollinated progenies not equivalent to half-sib families, or not obtainable.
(2) Early estimates of GCA not available from progeny trials.

selection. It must be noted that for actual seed orchards only a small proportion of the breeding population genotypes would be used, and that these orchards are not envisaged as a major source of selections for subsequent generations of the breeding population.

Finally, we can suggest a way in which SCA can be utilised within the general scheme which we have proposed. When new selections are introduced to the breeding

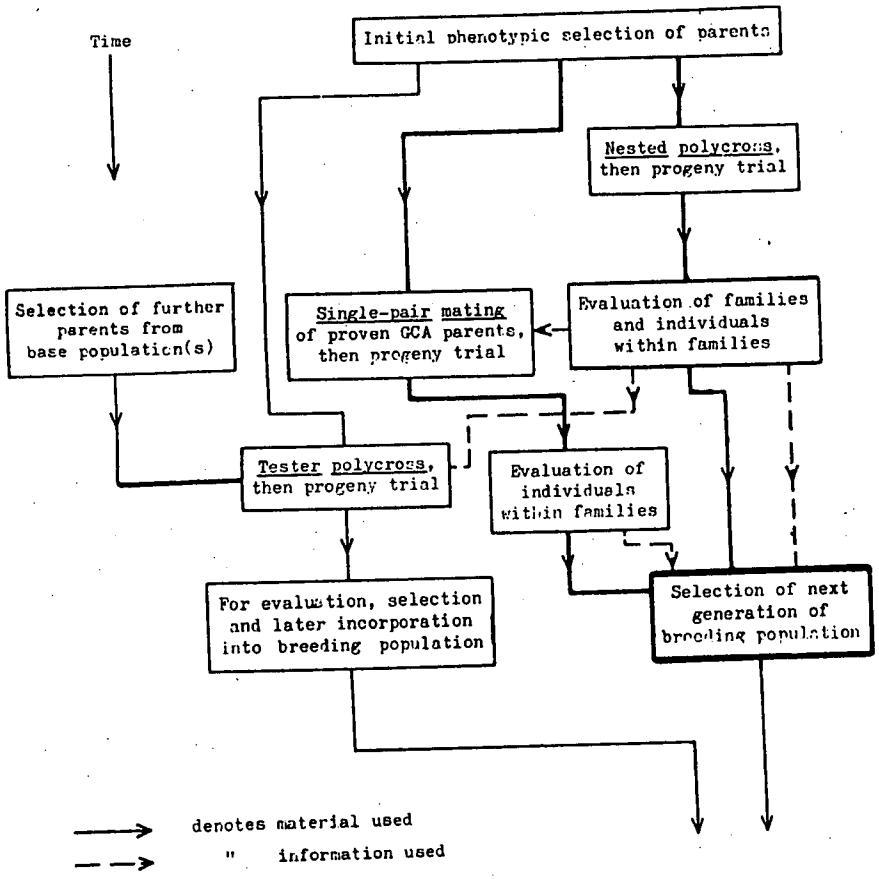



FIG. 5—Flow Chart 4. Suggested deployment of material in mating designs and use of information to produce advanced generation breeding populations

Conditions: (1) Wind-pollinated progenies not equivalent to half-sib families, or not obtainable.

(2) Early estimates of GCA available from progeny trial.



population by crossing with parent clones, the new selections can be derived from a population which combines heterotically with the original base population. Alternatively, the new clones could be selected for particular desired characteristics in the hopes that their less desirable characteristics could be later eliminated through genetic recombination. In one or both of these ways we hope to utilise some of the natural populations of radiata in our own breeding programme.

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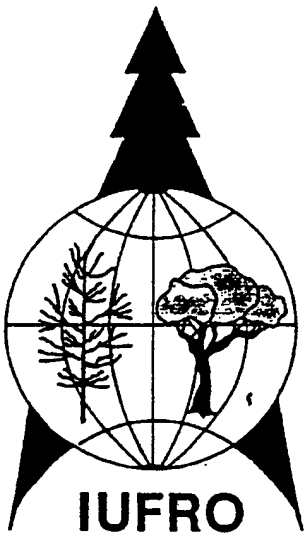


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# THIRD-GENERATION BREEDING STRATEGY FOR THE NORTH CAROLINA STATE UNIVERSITY-INDUSTRY COOPERATIVE TREE IMPROVEMENT PROGRAM

Steven E. McKeand and Floyd E. Bridgwater[1]

Abstract.--A strategy for the Cooperative's third-cycle breeding program for loblolly pine (*Pinus taeda* L.) was developed to provide maximum genetic gain in the short-term as well as to maintain genetic diversity so that long-term genetic gains will also be possible. Our strategy will be to manage a hierarchy of three populations, each at a different level of intensity. The **mainline population** will consist of 160 selections that are available to each cooperator (i.e. recruitment population) in a given geographic region. These populations will be managed as subdivided breeding populations (40 sublines of 4 trees each) primarily to provide for long-term genetic gain. The most intensively selected and managed hierarchy will be the **elite populations**. A highly selected group of trees (approximately 40 selections) will be managed to provide maximum short-term genetic gain for each member's program. A third hierarchy will be the **genetic diversity archives** managed to preserve genotypes with extreme breeding values for individual traits (not necessarily for all traits combined) as an insurance population for environmental or selection criteria changes in future generations. Each generation, decisions will be made as to the value of including these archive selections into the mainline population as well as to the value of breeding this population of trees.

The improved efficiency of this proposed breeding strategy along with the reduction in population sizes compared to our current program, will result in a substantial reduction in effort by individual cooperators. The increase in selection intensity used to reduce the population sizes and the increased rate of breeding made possible with fewer trees will substantially increase gains in the next generations. While the most intensive effort will be devoted to those populations providing immediate genetic and financial gain, the long-term well-being of the genetic resource will be maintained by judicious management of all three hierarchies.

Keywords: Elite breeding populations, genetic gain, mating designs, *Pinus taeda* L., testing designs

## INTRODUCTION

As the North Carolina State University - Industry Cooperative Tree Improvement Program progressed toward the third cycle of breeding, every effort was made to develop an efficient, cost effective breeding strategy to ensure both short- and long-term benefits for Cooperative members. A primary criterion for the breeding strategy was that it be flexible for current and future breeders. Decisions made in the third-cycle program will influence all future loblolly pine breeding within the Cooperative's working area, and options available to future breeders must not be constrained by the methods employed in the next cycle. In addition, the strategy is flexible in accommodating the diverse objectives of the membership. While the members share in supporting the mission and objectives of the Cooperative, there is a diversity in product goals, aggressiveness of programs, and investment level that must be considered. Additionally, as new information becomes available, the plan should be flexible enough to incorporate the information.

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Third-cycle breeding will commence around 1997 when 30-40% of the third-cycle selections are available. A summary of the breeding strategy adopted by the Cooperative is given in this paper. More details are available in the report prepared for Cooperative members (Anonymous 1992).

## MAINLINE POPULATION

The size of the mainline breeding population is the driving force behind many breeding strategy decisions. The appropriate breeding population size depends upon the trade-offs of short-term versus long-term gain. Short-term gain is maximized by intensive selection and, hence, small effective population size. Long-term gains are maximized by milder selection and larger population sizes. Population size basically affects the risk of losing favorable alleles through genetic drift, and this is a more serious problem for those alleles that are at a low frequency. The loss of alleles affects the progress possible in breeding programs. The problem of choosing population size basically "reduces to one of deciding on the minimum effective population size that will avoid the major effects of drift, that is, that will retain most of the genetic potential in the population" (Rawlings 1970).

Many simulation studies as well as long-term selection experiments indicate that effective population sizes ($N_e$) $\geq$ 50 are sufficient for maintaining low frequency favorable alleles in a breeding population. Long-term gain should be possible for many generations provided that $N_e \geq 50$ is maintained (Namkoong et al. 1980). For favorable alleles at frequency $\approx$ 0.10, a population size of $N_e = 30\pm$ gives a 95% probability that these alleles will be fixed at frequency = 1.0 at reasonable selection differentials (i.e. 1/100) (Rawlings 1970). Alleles at very low frequency (.01) would require a much larger population size with similar selection intensity to have a high probability of fixation (Kang 1979, Namkoong et al. 1988, Robertson 1960). Even when many of the simplifying assumptions (such as equal gene frequencies and only additive genetic effects) are relaxed (e.g. Baker and Curnow 1969, Mahalovich 1990), the genetic gain from populations with $N_e < 100$ are very comparable to gains from much larger populations.

For alleles that are not under selection (but may be valuable for changing selection goals in future generations), the population size necessary to maintain these alleles with a high probability is higher than for those alleles under selection (Kang 1979). Again, the appropriate $N_e$ depends upon the gene frequency and the desired probability of not losing the allele.

Namkoong et al. (1988) also emphasized the benefits of maintaining a relatively large population size in the first generations of breeding. Low frequency alleles will be selected and will move into the intermediate frequencies where future selection will be more effective in smaller populations. Fortunately, the Cooperative managed very large breeding populations in the first two cycles. Each cooperator had access to an average of 600+ selections for a given geographic area - over 4000 selections throughout the Cooperative.

Given the long-term nature of the Cooperative's breeding program and uncertainties about future selection pressures (Kang and Nienstaedt 1987), a third-cycle breeding population with an initial effective population size of $N_e$ = 160 selections available for each cooperator (i.e. a recruitment population) is reasonable. The traditional approach of keeping the breeding population as large as the breeder can afford is appropriate, and 100 to 200 selections is sufficient to yield long-term gain. While this population size is much smaller than the 600$\pm$ selections currently available to each cooperator, the reduction to 160 selections is warranted since 1) The mainline breeding populations will be composed of local selections only. For future generations, selections from adjoining or even distant geographic regions may be combined in breeding populations depending on the results from a large study designed to evaluate geographic variation among our plantation selection populations (Anonymous 1987). And, 2) The 600$\pm$ second-cycle selections will have been tested, so that estimates of breeding values for each tree will be available. Had we started with untested selections, 160 trees with poorly estimated breeding values would be a bigger risk and probably unacceptable.

Both parental and offspring selections will be used in the third-cycle breeding program. The top 160 clones based on breeding value estimates from current tests, and some procedure to limit coancestry will be utilized.

The number of sublines and the number of entries per subline is primarily dictated by the size of the breeding population and the number of unrelated clones needed to establish orchards. With 160 selections, and subline sizes of 4 clones, 40 sublines would be available for establishment of orchards or other types of production populations. There are several benefits from making the sublines as small as possible that include: 1) Inbreeding within a line will increase more rapidly, thus increasing the total genetic variance from which to make gain (Falconer 1989); 2) The 40 sublines allow for exploitation of the increased genetic variance (1 + F) among lines, since selections from only the best lines would be used for orchard establishment each generation; 3) Alleles can be fixed faster in small populations (Kang 1991); 4) If selection criteria change in future generations, small sublines will be more responsive to selection (Namkoong et al. 1988); 5) Breeding and testing each subline will move more rapidly with small sublines. Each can be managed as a discreet unit; 6) Small sublines can be combined into larger sublines in the future. If alternative population management strategies are deemed appropriate for future breeding cycles, the populations can be readily restructured; and 7) A minimum effective population size of 40 can be maintained even after many generations of breeding.

Some of the potential disadvantages of maintaining small sublines include: 1) Inbreeding may be too rapid so that alleles (both favorable and unfavorable) are fixed too rapidly and selection is not effective; 2) Inbreeding will reduce fecundity so that seed production for breeding and operational needs is reduced; 3) Recombination is restricted to within lines in the breeding population; and 4) Sublines will be eliminated due to genetic sampling, drift, and/or selection.

A sublining system is not only effective for managing inbreeding, but it is also very effective for managing a series of small, homogeneous populations of trees ordered along a continuous environmental gradient. There are no discrete "breeding regions" in the Cooperative, but a continuous series of populations. Since environmental factors which affect genotypic performance are continuous, different selection criteria can be utilized in a continuous fashion in managing the sublines. For example, the importance of fusiform rust resistance gradually changes both N-S and E-W. The economic weight for rust resistance can be changed in a selection index for sublines occurring along rust gradients). Sublining also simplifies breeding, since each cooperator will manage a given set of sublines. The logistical problems of exchanging selections, pollen, seeds, and seedlings for breeding, testing, and selection will be eliminated.

The mating design for the sublining system will be a complementary design that combines pollen-mix mating to estimate GCA's and control-pollinated matings for within-family selection. van Buijtenen and Burdon (1990) and Burdon and van Buijtenen (1990) emphasize the value of pollen mix designs combined with control-crossed designs. A pollen mix of 20$\pm$ average clones for each of the eight Test Areas (Anonymous 1984) in the Cooperative will be used. An adjustment factor will be calculated for each pollen mix based on standard check seedlots.

Control-pollinated families will be used only for within-family selection. With 4 parents in each subline, 6 crosses among them will be made. Seedlings from the control-pollinations will be planted in non-replicated full-sib 36-tree family blocks, and the best phenotypes will be selected. The crosses from which selections will be made will be determined by the results of the pollen mix tests. Crosses with the highest mid-parent value will be identified, and the best individual tree(s) within those crosses will be selected.

## ELITE POPULATIONS

The extra gain to be obtained from breeding only the most highly selected trees for specific geographic regions (e.g. Cotterill et al. 1988) was the main attraction to including elite populations into our hierarchy of breeding populations. There is a large increase in selection intensity by utilizing only the top clones in a recruitment population rather than all 160 clones. The average current recruitment population is about 600 clones (Anonymous 1982). In the mainline population, 160 of these clones will be utilized for a selection intensity of $i = 1.231$. In an elite population, the selection intensity for 40 out of 600 is 1.937 or 57% higher than for the mainline population. The selection intensity benefits derived from reducing the population size from 600± will occur only once, since populations are not likely to be reduced in size in future generations. Another major advantage of breeding elite populations is rapid generation turnover. It is much quicker to breed only 40 entries rather than the 160 entries in the mainline, thus gain per year will be higher (Mahalovich 1990).

There are numerous options available for management of elite populations. Rather than developing one elite population breeding strategy for all cooperators, elite populations will be customized for each cooperator. Individual cooperators or groups of cooperators will decide how their elite populations will be managed. Populations can be structured using local or non-local genotypes to utilize only general combining ability effects or both general and specific combining ability effects. Since one of the main benefits of elite populations is that breeding cycles can be completed more rapidly than in the mainline, any strategy adopted must have fast generation cycling as a feature.

One option we are evaluating is the use of inbreeding is to rapidly increase homozygosity and the frequency of favorable alleles as well as to increase additive genetic variance. Another advantage of very rapid inbreeding (e.g. selfing) is that the among-family selection intensity for selfed families is greater than for bi-parental crosses. With selfing, the best parent based on GCA performance is mated with itself and can be selected without reducing its breeding value by crossing it with another parent.

While selfing has potential to give the most rapid gain, there are concerns. Getting an adequate number of selfed seeds (200±) is difficult or impossible for many clones. Based on past selfing trials, selfing will only be feasible for about 50% of the clones. If an elite population size of 40 clones is utilized, seeds from about 20 clones can probably be obtained at a reasonable cost. Selfing may also increase homozygosity so rapidly that selection may not have a chance to operate. Slower inbreeding such as full-sib mating or half-sib mating could be more effective in the long run. For the second and future cycles in the elite population, there are numerous options available for advancing to the next generation. Selfing can continue and $S_2$ offspring from the selected $S_1$'s can be produced. Progeny from outcrossed trees could be selfed to create new $S_1$'s. For milder levels of inbreeding that might allow selection to operate more effectively, crosses between full-sibs ($\theta = .25$) or crosses among half-sibs ($\theta = .125$) can be made. There are also numerous options for crosses among all clones within the elite population (e.g. among $S_1$'s or among any selections within or among sublines would be possible).

Additionally, there is no reason to consider the elite populations as "closed". There will be overlap in breeding values between trees in the mainline and elite populations (i.e. heritabilities are relatively low and selection is far from perfect). Each cycle, the best genotypes from the mainline population in the same geographic area or from other elite and mainline populations can be infused into the elite population if the breeding values meet selection criteria. Infusion of new genotypes will also enhance the genetic variation in the elite populations.

If future breeders desire to maintain a sublining system within the elite population, related trees from within each diallel can be maintained within lines. The full-sib and half-sib crosses could be one way of maintaining the genetic integrity of a subline. In fact, the full-sib crosses described above can be considered as 2-tree sublines from within the 4-tree diallels. The selfs are nothing more than one-tree sublines if $S_2$'s are made. Flexibility for future options is a key advantage of this elite population management scheme.

## GENETIC DIVERSITY ARCHIVES

The need to conserve forest genetic resources has been discussed in a recent report by the National Research Council (NRC 1991). We have made no attempt to elaborate on the economic, biological, or ethical needs for gene conservation, but we recognize the need to manage the extremely diverse and rich gene pool for loblolly pine. The fully-pedigreed populations that we have developed and will continue to develop are an invaluable resource for breeders and forest scientists today and many generations from now. Unique genotypes must be preserved starting immediately if we are to insure the availability of this resource in the future.

For long-term preservation of genetic diversity in our loblolly pine populations, an additional management strategy (apart from the mainline breeding program) will be the judicious use of Genetic Diversity Archives to preserve all existing selections being bred in diallels as well as many future selections. First, archives or clone banks will be used to preserve the gene pool that the NCSU-ICTIP amassed in the 1950's through the 1980's. Approximately 4000 trees have been selected from natural stands and unimproved plantations throughout the Cooperative's working area (Maryland to Mississippi). The vast majority of selections from this enormous genetic sample still exists in seed orchards or clone banks. A concerted effort will be made to preserve these selections in the genetic diversity archives. If environmental or selection criteria change dramatically in the future, these archives will be valuable if mainline breeding populations do not contain the genes that breeders desire.

In future breeding cycles, rather than lowering the selection intensity in the mainline population to ensure the maintenance of rare alleles or rare allelic combinations, up to 150 "unique" genotypes (in addition to the 160 in the mainline) will be selected from each recruitment population for inclusion in the archives. These trees will have unique and extreme values for different traits (e.g. very large volume but relatively poor straightness or very high wood specific gravity and relatively poor rust resistance). If future breeders find that collection of open-pollinated seeds or additional crossing among clones in the archives is warranted, all trees will be available for this effort.

Genetic resources are also available from other loblolly pine breeding programs in the South and from around the world. The Western Gulf Forest Tree Improvement Program and the U.S.D.A. Forest Service both have advanced-generation breeding programs that are based on a different gene pool than the Cooperative's. Where loblolly pine has been used as an exotic (e.g. Argentina, Brazil, China, South Africa, Zimbabwe), there are also breeding programs and potential genetic resources. Natural populations of loblolly pine in the South will continue to exist for selection in the unlikely event that desirable alleles are not available in breeding or archived populations.

We have taken a very conservative approach to managing and preserving the genetic resources of our loblolly pine populations. Namkoong (1984) argues that hierarchical populations are not necessary for managing genetic diversity. The multiple population breeding strategy in itself is a gene conservation strategy. "As a part of breeding population management, gene conservation implies much more than preserving one generation's allelic array. In this context, gene conservation is identical to long-term breeding or populational gene management and implies wide population sampling as a start for designed multiplicity, and continued development of population diversity as environmental and economic demands change." (Namkoong 1984). We have elected to add the genetic diversity archives as a low-cost insurance of diversity.

## SUMMARY

The breeding and testing workloads for the Cooperative's 3rd-cycle program will be significantly smaller then the workloads in the current breeding cycle. We estimate that the number of crosses, including polycrosses, will be 50 to 130 per cooperator, or about 1/5 to 1/2 the number of crosses in the 2nd-cycle program. The testing program will consist of 7 to 15 acres of tests per cooperator, or about 1/10 to 1/5 the size in the 2nd-cycle. Every effort to keep the logistics as simple as possible in the field have been made. While the management and coordination of the different populations

(especially elite populations with potentially different breeding strategies) will be complex, the actual breeding, testing, and selection activities by cooperators will be much simpler than the diallel system we currently use.

The breeding strategy was developed without having made extensive genetic gain calculations for various breeding strategies. A program of high-priority research is planned to evaluate population structure and effective population size issues. Alternative theories of population management will be evaluated by computer simulation, and the most promising of these will be tested in model breeding populations. Model populations will be an integral part of our program's research effort since the information is required to guide our future breeding.

A final issue concerns the compatibility of the third-cycle breeding plan with new technologies in forestry and genetics. If, for example, vegetative propagation of mature loblolly pine becomes feasible, breeders may want to reconsider breeding for specific combining ability as well as for general combining ability in the mainline program. The small sublines lend themselves very well to a paired sublining strategy or a reciprocal recurrent selection strategy to advance SCA and GCA (McKeand et al. 1986). Another option available to any cooperator is to advance SCA and GCA in elite populations.

Vegetative propagation could also change and enhance the testing and selection program. If individuals within families can be clonally replicated, the within family heritability would be much higher, and gain from within family selection would be increased. Additionally, to exploit SCA and GCA more fully in production populations, additional crossing among clones and planting of these crosses in replicated field trials may be desirable.

New developments in molecular genetics can also be incorporated into the Cooperative's breeding strategy. If molecular marker aided selection (MAS) for quantitative traits becomes feasible (we are currently collaborating with our molecular genetics group on a large experiment to test the techniques), the line breeding and particularly the inbreeding in the sublines will be a major benefit. Marker aided selection depends upon the maintenance of linkage disequilibria between the marker loci and the quantitative trait loci (Lande and Thompson 1990). Line breeding as opposed to random mating among individuals is very effective at maintaining linkage disequilibria for multiple generations, and enhancing the efficiency of MAS.

For other biotechnologies such as recombinant DNA to be applied to forest tree populations, breeders must have a better understanding of the genetic control of specific traits. The elite populations lend themselves to very intensive genetic experiments where breeders can intensively study a few, highly selected individuals to conduct the necessary physiology and biochemistry experiments.

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# Expected efficiencies of mating designs for reselection of parents

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Expected efficiencies, in terms of genetic gain from reselection of parents (backwards selection), were compared for hierarchical mating, factorial, partial factorial, modified half diallel, and partial diallel crossing designs and polycrosses; this was done in parallel with a separate study of expected efficiencies for advanced-generation (forwards) selection, assuming a fixed-resources model of 100 parents and 10 000 offspring. The present study considered a single-trait case, with variable numbers of crosses per parent, varying heritability ($h^2$) levels ($h^2$ = 0.1, 0.2, and 0.5), and varying ratios of specific combining ability to general combining ability variance (0, 0.5, and 1). Compared with the case of forwards selection, the relative efficiencies of the different designs were generally similar. Two notable exceptions were the comparative inefficiency of small, disconnected factorial sets for backwards selection and the generally high (but not always maximal) efficiency of polycrosses for this purpose.

BURDON, R. D., et VAN BUIJTENEN, J. P. 1990. Expected efficiencies of mating designs for reselection of parents. Can. J. For. Res. **20** : 1664–1671.

Les performances attendues, en terme de gain génétique obtenu de la resélection des parents (sélection récurrente), sont comparées pour des modèles de croisement hiérarchiques, factoriels, factoriels partiels, semi diallèles et diallèles partiels modifiés, et polycross. Ces travaux ont été réalisés en parallèle avec une autre étude sur la performance estimée dans la sélection de générations avancées (nouvelle sélection), en assumant une situation avec un matériel fixe de 100 parents et 10 000 descendants. L'étude porte sur un seul caractère stable avec des nombres variables de croisements par parents, avec des niveaux d'héritabilité ($h^2$) variables ($h^2$ = 0,1, 0,2 et 0,5) et différents rapports entre l'aptitude spécifique au croisement et la variance de l'aptitude générale à ce dernier (0, 0,5 et 1). Comparativement à la nouvelle sélection, les performances relatives des différents modèles étaient généralement semblables. Il y avait deux exceptions importantes : la performance comparativement faible avec des petits groupes factoriels non reliés dans le cas de la sélection récurrente et la performance généralement élevée des croisements polycross dans ce contexte.
[Traduit par la revue]

## Introduction

Expected efficiencies of alternative mating designs, for giving genetic gains in advanced-generation (or forwards) selection, was analyzed by van Buijtenen and Burdon (1990). It was shown that provided designs were balanced in representation of parents and provided the parents represented a random sample of potential selections, a variety of designs giving full-sib family information differed very little in gain efficiency for a given number of crosses per parent genotype.

As was pointed out, mating designs may need to serve several purposes that might all have to be considered in choosing designs. Relative efficiencies for the various purposes may therefore need to be considered jointly for purposes of decision making. For minimizing allele loss in breeding populations, Kang and Namkoong (1979, 1980) concluded that balance (equal representation of all parents) was the paramount consideration. For estimating genetic parameters, Namkoong and Roberds (1974) made a comprehensive comparison of designs, although this function could often become less important for the next generation or so (Namkoong 1970) as genetic parameters would often become reasonably well known. For testing or reselection of parents (backwards selection), work has also been done (van Buijtenen 1976; Lindgren 1977; Pepper and Namkoong 1978), but it remains of interest to compare designs in situations parallel to those in which they are compared for advanced-generation selection. Accordingly, the present paper compares mating designs for reselection of parents in the same situations as covered by the companion paper by van Buijtenen and Burdon (1990).

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## General method

As described by van Buijtenen and Burdon (1990), selective efficiency is considered in terms of genetic gain for given numbers of crosses per parent. For the present study, however, mass selection is not a relevant alternative against which other options might be compared, so expected gains are calculated as absolute values per unit selection intensity.

Expected gains are formulated according to the modified model of Osborne (1957), as described by van Buijtenen and Burdon (1990). Briefly, expected genetic gain ($\Delta G$) is expressed as

$$[1] \quad \Delta G = i\sqrt{V_I}$$

where $i$ is the intensity of selection (reselection) in standard deviations and $V_I$ is the variance of the selection index. In subsequent derivations, the following coefficients are used:

- $d$ = number of trees per plot
- $f$ = number of females
- $k$ = number of crosses in which a parent is involved (in diallel)
- $l$ = number of locations
- $m$ = number of males

$p$ = number of parents in diallel
$q$ = total number of parents
$r$ = number of replications
$t$ = number of sets
$u$ = number of females/number of males in factorial

Now the index, $I$, is for the single-trait case of the general form

$$[2] \quad I = \sum_z h_z^2 \Delta z$$

where $\Delta z$ in this case is the effect of a seed parent in the progeny test classification, or of any group or subgroup within which the parents are nested and $h_z^2$ is the heritability of the effect in question. Because the selection in this case is not being done within the offspring of the parents in question, within-family genetic sampling effects contribute nothing to the numerators of the relevant heritabilities (cf. van Buijtenen and Burdon 1990).

For $V_I$ we have

$$[3] \quad V_I = \sum_z (h_z^2)^2 s^2 \Delta z$$

where $s^2\Delta z$ = variance of effects within category $z$. In turn

$$[4] \quad s^2\Delta z = \frac{MS_z}{N_z} \frac{df_z}{df_z + 1} = \frac{SS_z}{N}$$

when $z$ denotes sets of parents.

$df_z$ = degrees of freedom of clones (or subclasses) for category $z$
$MS_z$ and $SS_z$ = mean square and sum of squares, respectively, for category $z$
$N_z$ = number of individuals per class or subclass for category $z$
$N$ = total number of individuals in the progeny test classification

However, $s^2\Delta z$ equals $4SS_z/N$ when general combining ability (GCA) information on individual parents is being used, since GCA information reflects only half the additive genetic value of a parent.

## The progeny test situation

The following situations are assumed (using the arbitrary convention that rare-parent classes are designated male and common-parent classes are female, although males can be equal in number to females):

(*i*) hierarchical or nested design, with $m$ males each mated to a different set of $f$ females
(*ii*) factorial designs:
  (*a*) complete factorials, with $m$ males crossed with each of $f$ females within each of $t$ disconnected sets
  (*b*) partial factorials, with $f$ females each involved in $k$ crosses with members of a group of $m$ males within $t$ disconnected sets

for balance, $f = um$, $u > 1$, $f$ and $m$ being integers

(*iii*) diallel designs
  (*a*) modified half diallels, without reciprocals or selfs with $p$ parents, within each of $t$ disconnected sets
  (*b*) partial diallels, with each of $p$ parents involved in $k$ crosses ($k < (p - 1)$) within each of $t$ disconnected sets
(*iv*) polycrosses, with each of the $q$ parents pollinated with a mix from a large number of pollen parents

Note that there are $d$ trees represented in each individual cross. Limiting cases are the following:

$f$ = 1 in hierarchical cross, single-pair cross
$p$ = 2 in modified half diallels, single-pair cross
$k$ = 1 (therefore $p$ = 2) in partial diallels, single-pair cross
$t$ = 1, single connected sets
$k = p - 1$, ordinary modified half diallel as special case of partial diallel
$k = um$, factorial sets complete, instead of partial

Assumptions made for present purposes include the following: (*i*) regularity of designs; (*ii*) equal numbers of individuals ($d$) per cross within each design; (*iii*) full randomization of individual offspring; (*iv*) all parents belonging to a single random population of possible selections, hence, (*a*) male and female groups have a common between-parent genetic variance (i.e., $\sigma_f^2 = \sigma_m^2 = \sigma_g^2$, where $\sigma_m^2$ and $\sigma_f^2$ are the variances among male and female half-sib families, respectively, and $\sigma_g^2$ = GCA variance, which is assumed to equal one-quarter the additive genetic variance ($\sigma_A^2$)) and (*b*) any genetic variance among disconnected sets will derive from purely random genetic sampling error, so the two between-sets variance component ($\sigma_{sets}^2$) equals zero.

From these assumptions, follow the analyses of variance already presented by Burdon and van Buijtenen (1990, Table 1). These analyses entail three variance components not yet mentioned here: $\sigma_w^2$ (within (full-sib) cross variance), $\sigma_s^2$ (specific combining ability (SCA) variance), and $\sigma_{w'}^2$ (variance within half-sib crosses (e.g., polycrosses)). The derivation of expected gains for specific designs is contained in the Appendix as follows: hierarchical crosses; factorials; diallels; polycrosses.

## Results

Expected gains from different mating designs are shown in relation to crosses per parent, for the various combinations of heritability and $\sigma_s^2/\sigma_g^2$, in Fig. 1. The impact of substantial SCA relative to GCA is clearly evident in the numbers of crosses needed per parent to approach maximum gains (Fig. 1, Table 1). Where $\sigma_s^2 = 0$, the partial mating designs showed maximal gains as soon as $k > 1$; in other words, in all cases except single-pair mating.

Polycrosses were consistently efficient in the presence of SCA, but were appreciably suboptimal with zero SCA and low heritability. Indeed, the numbers of crosses per parent required for control-cross designs to match the efficiency of polycrosses tended to be quite low, unless $\sigma_s^2/\sigma_g^2$ and $h^2$ were comparatively high (Table 2).

Small disconnected factorial sets tended to be appreciably less efficient than the corresponding diallel sets (Fig. 1, Tables 1 and 2). However, where factorial sets are larger (i.e., $m$ and $f$ larger), the factorials compared much more favorably; a single partial factorial (not shown in Fig. 1) consistently approached to within 0.5% of the expected gain from a single partial diallel. This general feature of the comparisons between factorials and diallels is further illustrated in Table 3 when $k$, the number of parents per set, and number of sets, are traded off to give a constant 50 crosses per set. Where $k$ is larger, the set sizes are correspondingly reduced and the factorials show poorly.

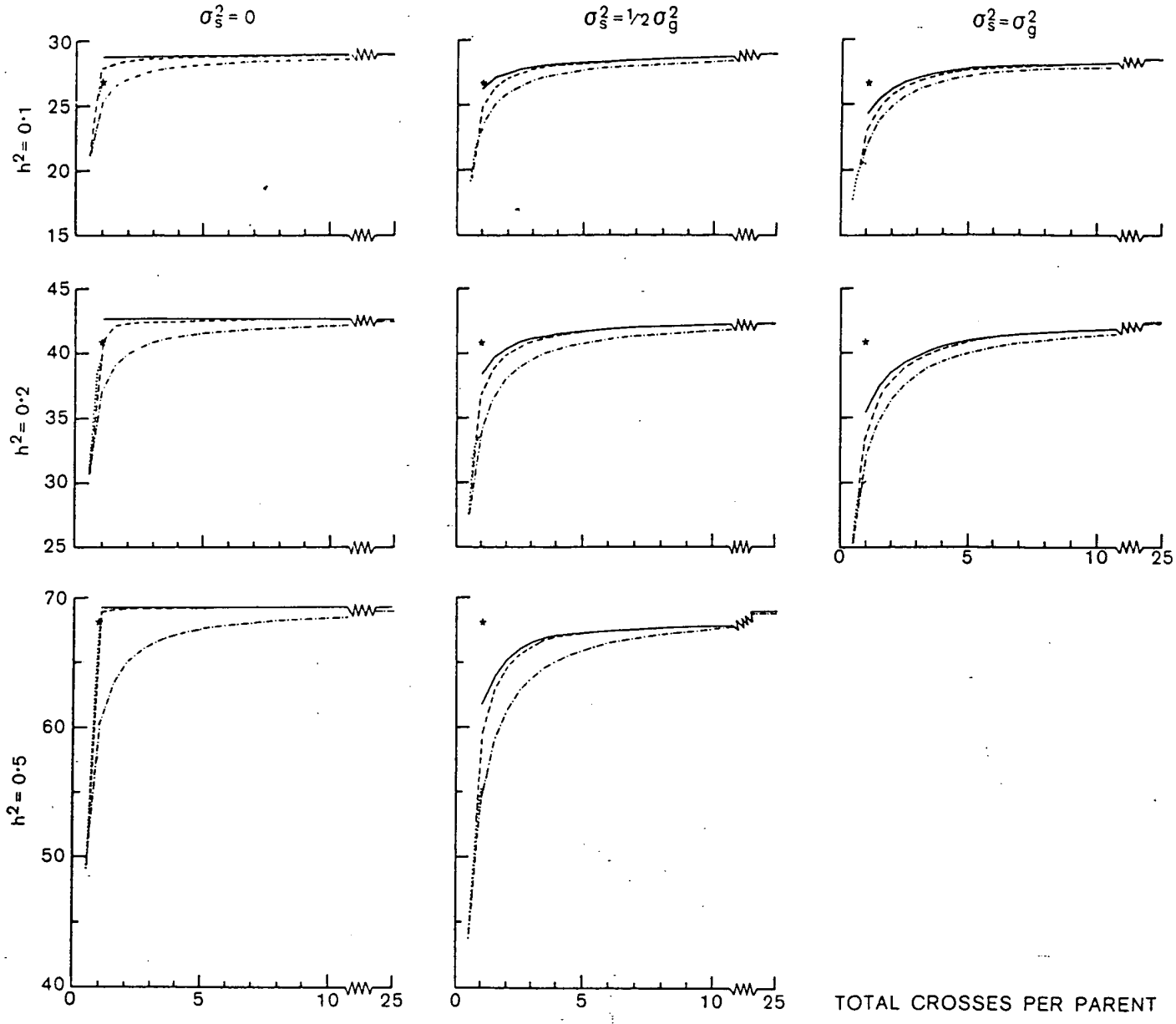


FIG. 1. Comparisons of expected gains per unit $i$ from different designs, under different combinations of $h^2$, $\sigma_s^2/\sigma_g^2$, in relation to total crosses per parent. Designs considered are hierarchical (·····), disconnected modified half diallels (-----), single partial diallel (——), and disconnected balanced factorials (·-·-·). Asterisks denote polycrosses. Sets are assumed to be fully cross-referenced.

The case of the single-pair cross was always substantially suboptimal, particularly as $\sigma_s^2$ increased. Even so, with the low value of $h^2$ (0.1) and zero SCA, the expected gain from single-pair crosses, under the conditions assumed, was 79% of that from polycrossing.

Hierarchical crosses tended to be decidedly inefficient, unless $f$ was large and $\sigma_s^2/\sigma_g^2$ was small (Figs. 1 and 2).

The significance of cross-referencing sets, which amounts to being able to include a term for sets in each of the relevant equations, is also illustrated in Table 4. Where sets are small, the contribution of such cross-referencing is clearly important. It is also slightly more important with lower heritability and with the higher ratios of $\sigma_s^2/\sigma_g^2$. A further illustration of the impact of set size is given in Table 5, for factorials in which total crosses per set are fixed at differing numbers but with no cross-referencing between sets.

Figure 3 illustrates the significance of imbalance between numbers of males and females for different cases of factorial crosses and hierarchical crosses. The reduction in expected gain was greatest with the intermediate level of imbalance, but imbalance increases set size in relation to crosses per parent and thus reduces the practical scope for cross-referencing sets. For hierarchical crossing, however, increasing imbalance ($f$ large) improved efficiency (Fig. 2), but this design was generally inefficient compared with the others, particularly when SCA was present.

## Discussion

### *Validity and robustness of results*

A study of this sort must depend largely on the assumptions made. Most of the assumptions have already been discussed by van Buijtenen and Burdon (1990). However, some are likely to be more critical for backwards selection than for forwards selection and are accordingly considered again here.

TABLE 1. Approximate genetic gains, as a percent of those realisable with 25 crosses per parent, with 1, 2, 3, and 4 crosses per parent for the cases where $\sigma_s^2 = \sigma_g^2$

| Mating design | Total crosses per parent (i.e., $k/2$) | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Partial diallel | 84 | 91 | 94 | 96 |
| Partial factorial | 84 | 91 | 94 | 96 |
| Disconnected diallels | 79 | 89 | 93 | 95 |
| Disconnected factorials | 76 | 87 | 91 | 93 |

NOTE: Results are those cases where applicable sets are assumed to be cross-referenced with respect to genotypic value.

TABLE 2. Numbers of crosses per parent ($k/2$) required for various types of balanced mating design to exceed the efficiency of polycrossing, for various values of $h^2$ and $\sigma_s^2/\sigma_g^2$

| $h^2$ | $\sigma_s^2/\sigma_g^2$ | Type of design | | | |
|---|---|---|---|---|---|
| | | Partial diallel | Disconnected diallels | Partial factorial | Disconnected balanced factorials |
| 0.1 | 0 | 1 | 1 | 1 | 2 |
| | 0.5 | 1.5 | 2 | 1.5 | 2.5 |
| | 1 | 2.5 | 2.5 | 2.5 | 4 |
| 0.2 | 0 | 1 | 1 | 1 | 3 |
| | 0.5 | 2.5 | 3 | 3 | 5.5 |
| | 1 | 5 | 5.5 | 5.5 | 7.5 |
| 0.5 | 0 | 1 | 1 | 1 | 7 |
| | 0.5 | 7 | 7.5 | 8.5 | 13 |

Regular mating designs, without missing crosses, have been assumed throughout. Where each parent is crossed with only a few others (i.e., $k$ is small), the GCA estimates for the parents involved in missing crosses will be severely compromised, with potential effects on the overall efficiency of selection. The loss of information from missing crosses could be far more severe than in forwards selection, which leaves scope for within-family selection, except of course in the missing crosses themselves. The comparative robustness of small diallels and small factorial sets has apparently not been documented. However, small factorials sets ($m$ and $f$ small) seem inappropriate on grounds of inherent inefficiency, while it seems unlikely that large diallel and large factorial sets would differ materially in robustness of genetic gains.

Where $k$ is small it should be easier to approach the full number of intended crosses. In this connection, adopting small $k$ values should make it easier to circumvent in diallel difficulties where particular parents are unsatisfactory producers of pollen or female flowers. Factorial crosses automatically accommodate such flowering constraints.

For the partial designs it is assumed that the precision of estimating half-sib family means is as implied by the expected mean squares given in Table 1 of van Buijtenen and Burdon (1990). Some noteworthy results hinge on this assumption, so cross-checking by Monte Carlo runs is suggested as a follow-up study.

For polycrosses it is assumed that pollen parents are numerous and that they make equal contributions to the offspring. If numbers in pollen mixes are not large, then unequal contributions will clearly become much more important.

TABLE 3. Comparative expected gains from disconnected partial diallels and disconnected balanced factorials in which numbers of parents per set and total crosses per parent are traded off to give 50 crosses per set

| Crosses per parent ($k/2$) | Disconnected diallels | Disconnected balanced factorials | Parents per set |
|---|---|---|---|
| 0.5[a] | 27.52 | 27.52 | 100.0 |
| 1 | 38.18 | 37.89 | 50.0 |
| 1.5 | 39.20 | 38.72 | 33.3 |
| 2 | 39.64 | 38.96 | 25.0 |
| 2.5 | 39.82 | 38.93 | 20.0 |
| 3 | 39.87 | 38.77 | 16.6 |
| 3.5 | 39.84 | 38.52 | 14.3 |
| 4 | 39.75 | | 12.5 |
| 4.5 | 39.63 | | 11.1 |

NOTE: Sets are assumed not to be cross-referenced with each other, and $h^2 = 0.2$, $\sigma_s^2/\sigma_g^2 = 0.5$. Noninteger values of crossing parameters have been accepted.
[a]Single-pair crosses.

An absence of maternal effects has been assumed. Even if transient maternal effects exist, the breeder should use methods for correcting for such effects (cf. Burdon and Sweet 1976).

Also assumed is that prior selection has not truncated the genetic variation among parents. This means that the $\sigma_g^2$ values in the numerators in eqs. A1–A11 (Appendix) equal one-quarter of the base-population additive genetic variance. Some departures from this assumption will occur and will, in particular, increase the relative importance of $\sigma_g^2$. Adjustments for truncation, effectively revising eqs. 12–16 of van Buijtenen and Burdon (1990), should be possible.

The weightings given to male and female information in formulating the efficiencies of hierarchical crosses and unbalanced factorials (eqs. A1–A6, Appendix) is not so automatic as for forwards selection. The basis adopted, namely proportions of males and females, may be arguable, but other plausible alternatives seem unlikely to affect the general picture. However, imbalance would presumably increase sampling variation about the expectations.

A common time scale is implied for the different mating designs. However, any differences between designs in time required for completion will affect the comparative efficiencies in practical terms (Cotterill 1986).

*Comparisons among designs*

A consistent and predictable feature is that where $\sigma_s^2$ is appreciable relative to $\sigma_g^2$, high efficiency depends on crossing each parent with a number of others. Apart from the general limitations of hierarchical mating, the one respect in which type of pair-cross design seems important is the relatively low efficiency of small factorial sets. This reflects the fact that males and females are not cross-referenced against each other, except in terms of expected sampling deviations from the population mean.

A notable, and somewhat counter-intuitive, finding was the high efficiency, in the absence of SCA, of the partial designs where $k > 1$ but is still small. As mentioned earlier, this may warrant semiempirical checking.

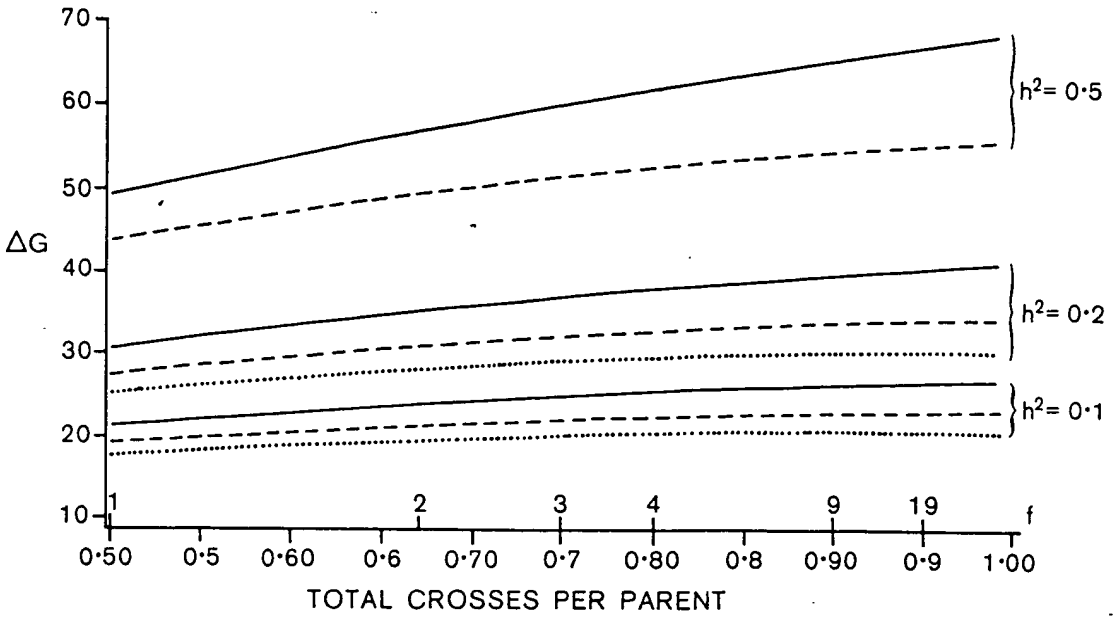


FIG. 2. Detailed plotting of expected gains from hierarchical crossing in relation to total crosses per parent and $f$, for different combinations of $h^2$ and $\sigma_s^2/\sigma_g^2$. —— denotes $\sigma_s^2 = 0$, ------- denotes $\sigma_s^2 = (1/2)\sigma_g^2$, ····· denotes $\sigma_s^2 = \sigma_g^2$.

TABLE 4. Expected proportion of potential genetic gain for varying $h^2$ and $\sigma_s^2/\sigma_g^2$

(A) Comparisons among sets sacrificed in disconnected modified half diallels

| | | No. of parents per set ($p$) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $h^2$ | $\sigma_s^2/\sigma_g^2$ | 3 | 4 | 5 | 10 | 20 | 50 | 100 |
| 0.1 | 0 | 0.788 | 0.853 | 0.887 | 0.949 | 0.978 | 0.994 | 1 |
| | 0.5 | 0.757 | 0.840 | 0.880 | 0.947 | 0.977 | 0.994 | 1 |
| | 1 | 0.734 | 0.830 | 0.874 | 0.946 | 0.977 | 0.994 | 1 |
| 0.2 | 0 | 0.804 | 0.861 | 0.893 | 0.951 | 0.979 | 0.995 | 1 |
| | 0.5 | 0.768 | 0.847 | 0.885 | 0.950 | 0.978 | 0.995 | 1 |
| | 1 | 0.742 | 0.836 | 0.879 | 0.949 | 0.978 | 0.995 | 1 |
| 0.5 | 0 | 0.815 | 0.867 | 0.897 | 0.953 | 0.979 | 0.995 | 1 |
| | 0.5 | 0.773 | 0.851 | 0.888 | 0.951 | 0.979 | 0.995 | 1 |

(B) Comparisons among sets sacrificed in disconnected balanced complete factorials

| | | No. of parents per set ($2m = 2f$) | | | | | |
|---|---|---|---|---|---|---|---|
| $h^2$ | $\sigma_s^2/\sigma_g^2$ | 4 | 6 | 10 | 20 | 50 | 100 |
| 0.1 | 0 | 0.811 | 0.892 | 0.944 | 0.977 | 0.994 | 1 |
| | 0.5 | 0.799 | 0.887 | 0.942 | 0.976 | 0.994 | 1 |
| | 1 | 0.790 | 0.883 | 0.940 | 0.976 | 0.994 | 1 |
| 0.2 | 0 | 0.816 | 0.896 | 0.946 | 0.978 | 0.995 | 1 |
| | 0.5 | 0.803 | 0.890 | 0.944 | 0.977 | 0.995 | 1 |
| | 1 | 0.793 | 0.885 | 0.942 | 0.977 | 0.995 | 1 |
| 0.5 | 0 | 0.820 | 0.899 | 0.947 | 0.978 | 0.995 | 1 |
| | 0.5 | 0.805 | 0.892 | 0.945 | 0.978 | 0.995 | 1 |

TABLE 5. Comparisons of expected gain from disconnected balanced factorials when numbers of parents and total crosses per parent ($k/2$) are traded off to give 50, 24, and 12 crosses per set

| Crosses per parent | Total crosses per set | | |
|---|---|---|---|
| | 50 | 24 | 12 |
| 1 | 37.89 | 37.02 | 35.30 |
| 1.5 | 38.72 | 37.35 | 34.58 |
| 2 | 38.96 | 37.07 | |
| 2.5 | 38.93 | | |
| 3 | 38.77 | | |
| 3.5 | 38.52 | | |

NOTE: Sets are assumed not to be cross-referenced with each other and $h^2 = 0.2$, $\sigma_s^2/\sigma_g^2 = 0.5$. Noninteger values of crossing parameters have been accepted.

Interestingly, it appears that the polycross can be appreciably less efficient than various pair-cross designs in the absence of SCA effects, particularly when heritability is low. Several factors contribute to this situation. Polycross families will have an inherently low signal to noise ratio with low $h^2$, unless $d$ is very large. At the same time, under polycrossing, each parent was effectively represented by only half as many offspring as under balanced pair-crossing schemes. Overall, the advantages of polycrossing seem less important than is widely supposed, unless SCA is substantial.

The range of possible regular designs was not exhaustive, but it should suffice to provide the requisite insights.

*Other issues in choice of design*

Depending on the situation, the breeder may want to be able to use the same trial for both forwards and backwards selection. Whatever the purpose in mind, it seems worth seeking a design (or combination of designs) that is efficient for both purposes. Many of the background considerations have already been covered by van Buijtenen and Burdon (1990), so only a few additional remarks are made here.

The main divergence in requirements for forwards and backwards selection arises in the comparative efficiencies of polycrosses and single-pair crosses, the former being

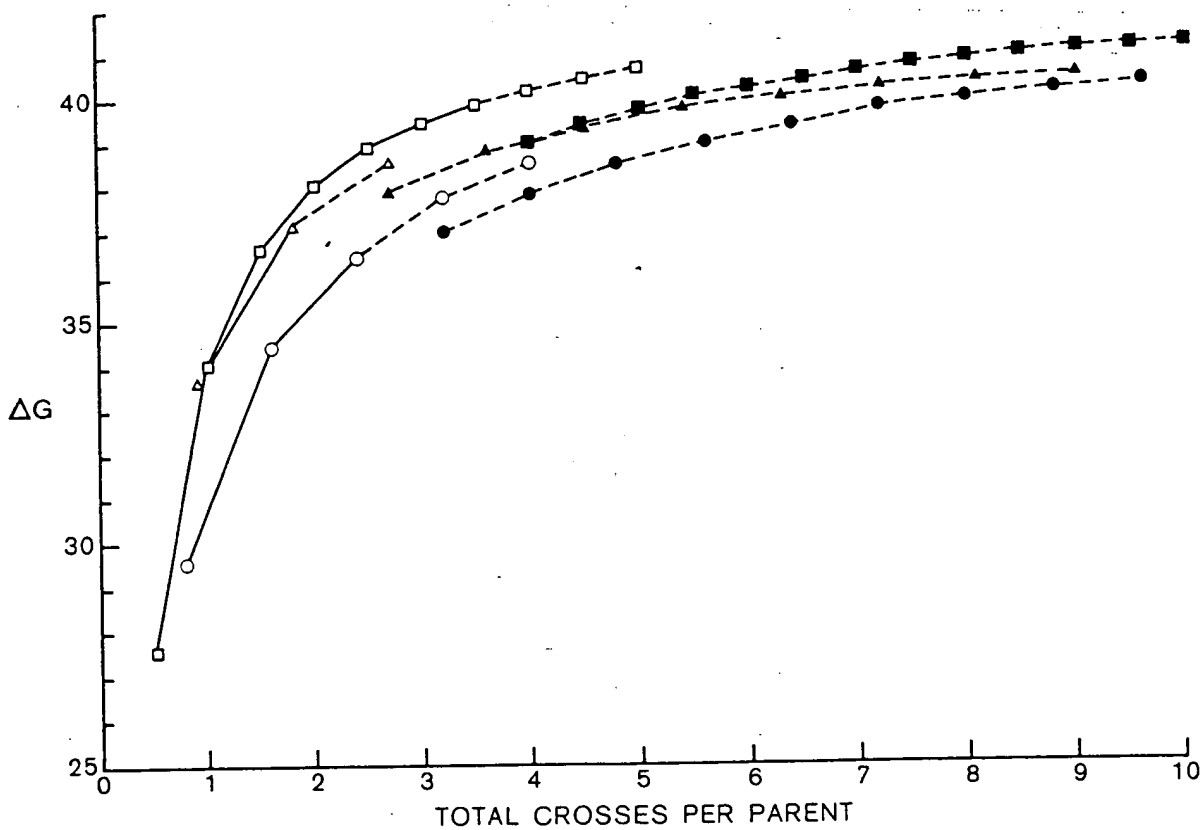


FIG. 3. Plottings, for case $h^2 = 0.2$, $\sigma_s^2 = (1/2)\sigma_g^2$, illustrating the impact of imbalance in complete factorials on expected gain. ■, □, $f = m$; ○, $f = 4m$; ▲, $f = 9m$, all for the case of sets being cross-referenced against each other. Solid symbols cover corresponding conditions with sets not cross-referenced against each other. Solid lines join points involving >50 crosses per set; broken lines apply otherwise.

suitable for backwards selection and the latter for forwards selection. Polycrossing is always near optimal for backwards selection, but unless $\sigma_s^2/\sigma_g^2$ is high, is in itself substantially suboptimal for forwards selection (van Buijtenen and Burdon 1990). It also has the major disadvantage, for forwards selection, of sacrificing full pedigree.

Overall, the presence of SCA is more important in choosing mating designs for backwards selection than for forwards selection. One other feature is that small disconnected factorial sets should be avoided for backwards selection, but larger partial factorial sets should be manageable.

Conflicts in requirements for forwards and backwards selection may become appreciable in field designs. For backwards selection individual randomization (single-tree plots) is the most efficient. For forwards selection, however, multitree plots may enhance the within-family component of selection.

## Acknowledgements

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**From:** Satish.Kumar@ensisjv.com
**Sent:** Thursday, July 20, 2006 1:40 PM
**To:** Lambeth, Clem
**Subject:** Re: Polymix breeding

Dear Clem,
It is great pleasure to hear from you. Following your leading paper on PMX/WPA, we recently completed a study to further explore this concept. We submitted a paper to the journal Tree Genetics and Genomes, which has been accepted subject to few minor changes. I am in the middle of preparing a revised version, which I should be able to send to you in about one week time.

In the mean time, I have attached here a reprint of our earlier paper on estimating relatedness using markers. I will be keen to pursue some similar projects in collaboration with you.

Kind regards,
Satish Kumar
Scientist (Tree Improvement)
Ensis-Genetics
(Registered office: New Zealand Forest Research Institute Limited)
Private Bag 3020
Rotorua
New Zealand

*(See attached file: molbreedpaper.pdf)*

"Lambeth, Clem" <clem.lambeth@weyerhaeuser.com>

**"Lambeth, Clem"** **<clem.lambeth@weyerhaeuser.com>**
19/07/2006 10:31

To<satish.kumar@ensisjv.com>
cc
SubjectPolymix breeding

Dear Dr. Kumar:

I have recently heard that you and some colleagues are doing work in an area dear to my heart, polymix breeding followed by paternity testing. Do you have any published or unpublished works that you could pass along (electronic versions would be appreciated)? I will be more than happy to honor any wish not to cite the work if it cannot yet be cited though citable works would be most helpful. I also have some new results in this area that may soon be written up for publication and any related work will be invaluable for the review section of the paper.

Many thanks for your any information you can provide.

Sincerely,

Clem Lambeth
Weyerhaeuser Company
Tree Improvement Program Manager
**Phone: 5016248510**
**FAX: 5016248505**

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ORIGINAL PAPER

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Robert Saich · Stephen Kenny · Gerald Tuskan

# Polymix breeding with paternity analysis in *Populus*: a test for differential reproductive success (DRS) among pollen donors

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**Abstract** Polymix breeding with paternity analysis (PMX/WPA) has been proposed as an alternative to traditional full-sib breeding and testing schemes. To fully capture the benefits of PMX/WPA, differential reproductive success (DRS) of pollen parents used in the polymix must be modest. DRS was evaluated in an operational test of PMX/WPA for a hybrid poplar breeding program. A 16-parent pollen polymix (*Populus nigra* L.) was used to pollinate seven clones of *Populus deltoides* (Bartr. ex. Marshall) under greenhouse breeding conditions. Progeny were grown out briefly and randomly sampled (357) prior to out-planting in field trials. Twenty-eight simple sequence repeat (SSR) loci were evaluated and 15 were selected for genetic characterization in small populations of three *Populus* spp (*P. nigra*, *P. deltoides*, and *P. balsamifera* spp *trichocarpa* Torr. & Gray). Seven loci were ultimately selected for paternity analysis of progeny. The average exclusion probability of the seven loci in *P. nigra* was 0.604; combined, the theoretical exclusion probability was 0.9999. However, only 95% of sampled progeny were unambiguously assigned a single paternal parent. Missing data likely accounted for most of the ambiguity. DRS was statistically significant though not prohibitive for practical utility of PMX/WPA as a breeding system. Of the 112 potential crosses in this study, 92 were represented. Eight of the 16 pollen parents contributed 83% of the progeny. Good pollen vigor, as measured by germination percent, did not ensure paternal success, but poor vigor was associated with lack of paternal success. PMX/WPA appears to be logistically and economically attractive for hybrid poplar breeding and testing.

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## Introduction

Polymix breeding with paternity analysis (PMX/WPA) of progeny has been proposed as an alternative to traditional full-sib breeding and testing schemes [4]. In short, PMX/WPA requires the application of pollen mixes of many male parents in controlled cross events, followed by paternity analysis of progeny using molecular markers. The approach offers good estimates of breeding values of parents and produces progeny populations with superior gain potential for forward selection. Other advantages of this approach include simpler and easier (thus, less expensive) breeding and testing programs, greater flexibility for inbreeding control, and convenient connections with other molecular genetic applications such as clonal fingerprinting and marker-aided selection [4]. These features are particularly relevant for trees, organisms which offer a number of obstacles to breeders (i.e., large, long-lived, and late-maturing habits requiring costly breeding and testing programs and facilities).

To fully capture the benefits of PMX/WPA, differential reproductive success (DRS) of parents used in pollen polymixes must not be high (most seed sired by only a few males). DRS in conifers (gymnosperms), though observed, is typically rather small (reviewed in Lambeth et al. 2001)

and insignificant relative to virtually all of the other factors controlling paternal success, such as amount and timing of pollen shed, location and distance of pollen donor relative to seed trees, and weather effects. Nakamura and Wheeler [8] concluded modest DRS in Douglas fir (*Pseudotsuga menziesii* Franco.) was likely due to genetic incompatibilities between male and female gametophytes (post-zygotic factors) but Nikkanen et al. [9] have demonstrated significant differences among spruce clones for pollen viability traits such as germination percentage and pollen tube growth rate. Such differences could arise from poor pollen vigor or genetic affects and could contribute to pre-zygotic competitive effects. Competition among male gametophytes has been well-studied in annual and non-woody angiosperms [7, 10, 11], but controlled studies with flowering forest trees are wanting.

The study described here results from the application of PMX/WPA for the creation of select hybrid poplar clones. Specifically, this study addresses the null hypothesis that all pollen parents in a polymix of pollens are represented equally in the progeny pool resulting from controlled inter-specific crosses with an array of seed parents (poplars are dioecious). Additionally, this study reports on the selection, optimization, and characterization of an array of micro-satellite simple sequence repeat (SSR) markers in three species of poplar.

## Materials and methods

### Plants, polymix construction, and controlled crosses

In the fall of 2003, multiple scions from each of seven select maternal *P. deltoides* Bartr. ex. Marshall (eastern cottonwood) clones originating from the upper mid-western United States, were grafted onto potted rootstock (*P. deltoides* var *siouxland*). In January of 2004, the grafts were moved into a greenhouse to encourage pistillate inflorescence emergence and receptivity. Similarly, in mid-January, branches bearing staminate inflorescences were collected from 16 select paternal *P. nigra* L. (black poplar) clones and placed in tap water baths in a greenhouse (15–18°C) to force pollen shed. All *P. nigra* clones originated from northern Italy; however, staminate cuttings were collected from an established clonal orchard located in eastern Washington. Seven of the 16 males had half-sib relatedness to one another (two pairs and a trio of half-sibs). Pollen was collected in glass beakers, funneled through 100 mesh screens to remove pollen catkins and poured into 20 ml glass vials (1/3 full) which were stoppered with cotton plugs. Vials were placed in desiccators containing silica gel and maintained at 4°C for the duration of the study (~1 week).

Upon completion of pollen collection, pollen germination trials were conducted by first rehydrating pollen aliquots for 2 h on dishes floating on a water bath in a hydration vessel and then, placing them on 1% agar containing 10% sucrose and minerals [1]. Pollen germination was scored after 18 h and reported as the proportion of pollen grains exhibiting pollen tubes equal to or greater than the diameter of the pollen grain. Individual clonal values were placed in categories (<10, 10–15, 15–20, and 20–25% germination). Beginning February 23, pollen mixes were created daily by combining equal weights of desiccated pollen from all 16 male clones. Prior to brush-applied pollination, the polymix was rehydrated for 2 h as described above. Individual inflorescences were pollinated once per day for 3 days and retained within protective paper bags. A single controlled-cross polymix family was created for each of the seven maternal *P. deltoides* clones. Seed capsules set and began to burst within ca. 30 days after pollination. Seeds were collected by family, weighed, and sown in flat trays. The rising crop (ca. 30 days after sowing) was transplanted into Ropac #3 trays (96 cells) in April, 2004, grown for ~30 additional days, and subsequently outplanted to a field test site near Pasco, WA. The hybrid poplar cross created here (*P. deltoides* × *P. nigra*) is one of the most common and valuable inter-specific hybrid taxa in the world and is recognized by the designation *Populus x canadensis* or *P. euromericana*. The hybrid is made with ease when *P. deltoides* serves as the female parent, though the reciprocal cross (*P. nigra* as female parent) virtually always fails [6].

### Molecular marker development

SSR markers were selected for this study because they are plentiful, co-dominant, and highly polymorphic in poplar. Though literally hundreds of SSR markers have been identified in poplar ([17] http://www.ornl.gov/sci/ipgc/ssr_resource.htm), few have been fully characterized and optimized for high throughput detection. One of the goals of this study was to identify and genetically characterize an array of SSR markers that were useful in three poplar species (*P. deltoides*, *P. nigra*, and *P. balsamifera* Torr. & Gray), the latter being a species native to the Pacific Northwest (black cottonwood) and common to hybrid poplar breeding programs.

*DNA extraction* DNA extraction was carried out on leaf tissue (progeny seedlings) using the DNeasy-96 Frozen Leaf Tissue Protocol following manufacturers instructions with tissue homogenization achieved via the Mixer Mill 300 (Qiagen, Valencia, CA) and from bud tissue (parents and population samples) using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following manufacturers' instructions. DNA quality and approximate quantity were assessed by comparing all samples against 50 ng of Lambda DNA standard on 0.8% agarose gels stained with ethidium bromide (EtBr) under UV light.

*SSR amplification and electrophoresis* A total of 28 candidate SSR primer pairs were evaluated (Table 1). SSR screening was achieved by amplifying approximately 1.5–2.5 ng of template DNA from *P. nigra* clones in a 10-μl final volume including 1× polymerase chain reaction (PCR) buffer, 2.0 mM $MgCl_2$, 0.4 μM of each dNTP, 0.4 μM of the forward and reverse primers, and 1 U of HotStarTaq DNA Polymerase (Qiagen, Valencia, CA). Amplifications were performed using a MJ Research PT-100

thermal controller with the following touchdown conditions: 15 min at 95°C, 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (3×), 94°C for 30 s, 52°C for 30 s, 72°C for 1 min (3×), 94°C for 30 s, 50°C for 30 s, 72°C for 1 min (29×) followed by a final extension of 72°C for 7 min. The amplification product was then diluted to a ratio of 1:50 (amplification:dd$H_2O$) and 1 µl of dilute amplification product was added to 10 µl of Hi-Di Formamide containing 1.2% GeneScan-500 [ROX] size standard. Samples were subsequently denatured at 95°C for 2 min and placed immediately on ice for 3 min before the sample plate was loaded on an ABI Prism 3,100 Genetic Analyzer for detection of SSR product. ABI software packages, GeneScan Analysis Software and Genotyper Software v 3.7, were used to visualize and evaluate alleles at each locus.

### Molecular marker characterization

Each of the 28 candidate SSR primer pairs were evaluated for use in determining paternity in each of the three poplar species using the criteria of genetic interpretability (i.e., does the primer pair produce a single locus that segregates in Mendelian fashion, and are the resulting alleles interpretable), heterozygosity level, exclusion probability, and expected null allele frequency. Primer pairs were initially screened using full-sib, controlled-cross family progeny of each species (five progeny each in *P. deltoides* and *P. nigra* and six progeny in *P. trichocarpa*). Based on inability to amplify and/or the presence of uninterpretable peaks, 13 of the 28 candidate primer pairs were discarded from further study. The remaining 15 primer pairs produced 15 loci that were determined to be potentially useful for paternity analysis. These primer pairs/loci were selected based on:

1. 16 progeny from each of the three *Populus* species (48 individuals; unrelated to individuals in the polymix study),
2. Eight progeny from each of six controlled-cross families, i.e., two families from each of the three species (48 individuals), and
3. 12 parent trees of the six families noted above.

Parental genotypes were confirmed by simple exclusion analysis of the paternal contribution to each progeny in each species family thus, providing a basis for the evaluation of each locus in the areas of interpretability and presence of null alleles. Data from each species were scored using Genotyper Software v 3.7. CERVUS 2.0 [5] was used to calculate heterozygosity, parental exclusion probabilities, and estimated null allele frequencies. Also, the number, size, and frequency of alleles were calculated for each locus.

### Paternity assignment and reproductive success

In August 2004, one leaf was sampled from each of ~50 randomly selected progeny from each of seven polymix derived families and from those pollen parents not previously genotyped in the marker characterization exercise. DNA extraction, SSR amplification, and electrophoresis followed protocols noted previously. Paternity assignment for each progeny was determined using programming and formal language (PFL), a computer program for estimating pollen flow using paternity exclusion and SSR markers (http://www.fsl.orst.edu/pnwtirc/publications/pnwtirc_pubs_date.htm). PFL allows for exclusion calculated on the basis of one or more locus genotype mismatches. All crosses were evaluated at both one and two genotype mismatch levels. Missing data were accommodated in the program. Where two or more paternal parents could have potentially sired a progeny, partial paternity was assigned (e.g., two potential pollen parents=0.5 each). A test for deviation from expected reproductive success across all females was performed using chi-square analysis.

## Results and discussion

### Pollen germination and seed set

Date of pollen shed varied from February 3 to 17, with individual clones shedding pollen for up to 7 days. In general, pollen germination rates in this study were low, ranging from less than 10% to around 25%. Pollen germination rates of 10–55% are considered sufficient for adequate seed set in most controlled crosses [13]; it is unknown whether variation in germination percent might contribute to pollen competitiveness in polycrosses of poplar. In the few controlled crosses performed with single-clone pollen lots exhibiting low pollen germination percent (<10%) in this study, seed set was nil, and inflorescence development was abnormal. Four of the 16 pollen lots tested (25%) had germination scores of <10%. There was no relationship between date of pollen shed and germination percent ($r$=0.04).

Seed set was generally very good. Estimated seed set for polymix crosses exceeded 3,000 for six of the seven female *P. deltoides* clones (680–4,860 seeds), while 20 seed weights ranged from 0.0083 to 0.0150 g. The maternal clone with a few seeds also had the lowest number of inflorescences to pollinate.

### Molecular marker characterization

Thirteen of the selected SSR primer pairs produced ambiguous peaks or failed to yield an amplified product (Table 1). Fifteen SSR loci from the remaining 15 primer pairs were characterized for the three poplar species studied. Most loci were highly polymorphic in all three species: the average number of alleles per locus was 8.6, 10.7, and 11.1 for *P. nigra*, *P. deltoides*, and *P. balsamifera*, respectively. Across all species, 368 alleles were detected at the 15 select loci (mean=24.5 alleles per locus; range=11–41; Table 1). In general, relatively few alleles were shared among species, particularly between either

**Table 1** Microsatellite (*SSR*) primer pairs evaluated for use in poplar paternity analyses are listed along with ABI dye used for detection, forward and reverse primer sequence, *SSR* motif, primer pair utility (*U* Used in study, *I* discarded due to uninterpretable band patterns (i.e., non-specific amplification, allelic dropout), *P* discarded due to poor amplification), number of alleles detected, and number of alleles shared among species, disregarding nulls (*DP. deltoides*, *NP. nigra*, *TP. balsamifera*)

| Primer Name[a] | Dye | Forward primer | Reverse primer | Motif | Utility | Number of Alleles | | | | Number of Alleles Shared | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *D* | *N* | *T* | Total | DN | DT | NT | DNT |
| ORNL 029 | FAM | TGGTGATCCAGTTTTGGTGA | GTCCTTGCAAGCCATGAA | AC | U | 4 | 11 | 5 | 20 | 0 | 0 | 0 | 0 |
| ORNL 127 | FAM | TCAATGAGGGGTGCCATAAT | CTTTCCACTTTTGGCCCTTT | TG | U | 10 | 3 | 4 | 17 | 0 | 0 | 0 | 0 |
| PMGC 14 | FAM | TTCAGAATGTGCATGATGG | GTGATGATCTCACCGTTTG | CTT | U | 2 | 7 | 7 | 14 | 0 | 0 | 2 | 0 |
| PMGC 420 | HEX | ATGGATGAGAAATGCTTGTG | ACTGGCACACTCTTTAACTGG | GA | U | 4 | 3 | 10 | 16 | 0 | 1 | 0 | 0 |
| PMGC 433 | TAMRA | GCAGCATTGTAGAATAATAAAAG | AAGGGGTCTATTATCCACG | GA | U | 15 | 8 | 14 | 23 | 5 | 8 | 5 | 4 |
| PMGC 576 | HEX | GCTGTCTAACATGCCATTGC | AATTTACATTTCTTTATCATCACC | GA | U | 13 | 16 | 17 | 41 | 2 | 0 | 3 | 0 |
| PMGC 649 | FAM | CATCCATGATATCAAACCAAATTAG | TGTAATCCAAACATAAAATCCCAAG | GA | U | 17 | 7 | 19 | 32 | 4 | 7 | 1 | 1 |
| PMGC 2011 | HEX | TCTACGAGGAAAGGGAAGGG | CTTTATAATGCATCATAAAGTTC | GA | U | 13 | 9 | 8 | 21 | 2 | 6 | 2 | 1 |
| PMGC 2221 | HEX | GCTTTGGGAACATGCTTAT | CCAAATCTCCAATTTTATGG | ATT | U | 19 | 15 | 21 | 40 | 5 | 6 | 7 | 3 |
| PMGC 2235 | FAM | GCCAAAATAGTAAGTGTGATGG | CACACATTCTCTCATTCAAAGC | GA | U | 15 | 9 | 16 | 31 | 0 | 8 | 1 | 0 |
| PMGC 2571 | FAM | TCTCGCAGATTCATGTAACCC | GACTGTATGTTGACCATGCCC | GA | U | 1 | 1 | 9 | 11 | 0 | 0 | 0 | 0 |
| PMGC 2675 | FAM | CACACCGACAAATTATGAGTG | TTTTAGAGTGAATTTTCCTGCG | GA | U | 18 | 10 | 14 | 32 | 1 | 7 | 2 | 1 |
| PMGC 2804 | FAM | AAAGTTTTTCATTTTCAATCCTTG | TAATCGCCTATACACAGGCG | GA | U | 8 | 7 | 12 | 24 | 0 | 2 | 1 | 0 |
| PMGC 2885 | FAM | CATGATCAAATTGGATTTGAATG | AAAGATGAACATGGCTAGCTC | GA | U | 10 | 8 | 7 | 21 | 0 | 4 | 0 | 0 |
| WPMS03 | HEX | TTTACATAGCATTTAGCCTTTAGA | CCAAATCTCCAATTTTATGG | ATT | U | 10 | 14 | 3 | 25 | 0 | 1 | 1 | 0 |
| AG1 | HEX | CTTGTAATTAAGAGCAAGCCA | ATGTTAAACTACCTCAAACATATCC | AT | P | – | – | – | – | – | – | – | – |
| ORNL 015 | HEX | CGTGAGTTTTGAGGCCATTT | CATGGAAAGGATCACCCACT | [AT]14 | I | – | – | – | – | – | – | – | – |
| ORNL 203 | FAM | CCACCAGGCATGAGATATGA | TCAAACCGAAAGGTCAACAA | [TA]4 | I | – | – | – | – | – | – | – | – |
| ORNL 206 | HEX | CCGTGGCCATTGACTCTTTA | GAACCCATTTGGTGCAAGAT | [GCT]7 | I | – | – | – | – | – | – | – | – |
| ORNL 286 | HEX | TCAGGCAGAAGGGTAGAGGA | CCTGACCCTGCTTGCTTATC | [GT]4 | | | | | | | | | |
| [GA]8 | I | – | – | – | – | – | – | – | – | | | | |
| PMGC 603 | FAM | GTACCTATGAAAGTAGGCAACAC | TTTTTTATCACTATCTCAGATAC | GA | I | – | – | – | – | – | – | – | – |
| PMGC 684 | FAM | GAAATTGAATATCTCTCACTTACC | TAATACGTGAAAAGTCAGGTTTTG | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2140 | HEX | GCTGTCAGAATCAAACACTTC | AAGCAGATAACTAAGACATGCC | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2156 | FAM | GATCTCTCTTACATCACTCATC | GAATGTCTTTACTCCATTGTTGG | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2515 | FAM | GAAAAGGGATTGTTAATAAACCC | CCAAAATCATAAAAGACAGGGC | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2585 | HEX | ACTGCTGTGTATTGCCCTAG | TAGTTGAAGTTGGAGCACAAC | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2610 | HEX | AACACGCAAGAACATACATAAG | GATTAACATGTTTCGCTACGC | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2862 | HEX | TTTGTAACTAATGAAGATTTGTAC | ATTTTTGTTCTTTTAACCAAAATTC | GA | P | – | – | – | – | – | – | – | – |

[a]Primers used in this study were provided by Dr. Steven Strauss, Oregon State University and Dr. Jerry Tuskan, ORNL. Primer development was as follows: ORNL markers: [15] PMGC markers: http://www.ornl.gov/sci/ipgc/ssr_resource.htm; WPMS markers: [12, 16]; AG1 marker: [2]

North American species (*P. deltoides* and *P. balsamifera*) and the European native *P. nigra* (5.2 and 6.8% alleles in common, respectively). *P. deltoides* and *P. trichocarpa* shared 13.6% of their alleles. Only 10 alleles (2.7%) were shared by all three species. Null alleles were detected in controlled crosses for six loci but were not considered shared between species because they likely do not represent the same mutational event. The mean allele frequency of the most common allele, across all 15 loci, was 0.308, 0.342, and 0.444 for *P. trichocarpa*, *P. deltoides* and *P. nigra*, respectively. Allele sizes and frequencies of all 15 loci evaluated are provided elsewhere (Appendix 1: Gene frequencies: http://www.ornl.gov/sci/ipgc/poplar polymix genefreg Appendix1.xls) Seven of the 15 loci characterized were ultimately deemed to be "informative" for all three species (Table 2) and were used for paternity testing in this study. For the seven selected loci, average observed heterozygosity was 0.681, 0.700, and 0.623 for *P. trichocarpa*, *P. deltoides*, and *P. nigra*, respectively. Similarly, the average exclusion probability for any one locus, given that the maternal genotype was known, was 0.754, 0.704, and 0.604. Although the Italian sample of *P. nigra* was the least genetically diverse of the three species evaluated, it theoretically possessed sufficient diversity to distinguish among all paternal parents. In the final analysis of all progeny and parents, the paternal exclusion probability for all seven loci combined was 0.9999. Measures of diversity (number of alleles, heterozygosity) observed for this breeding population of *P. nigra* were slightly lower than those detected in a previous study characterizing a range-wide collection of 23 individuals using nine SSR loci [16].

## Genotyping

Multi-locus genotypes were obtained for 357 hybrid poplar progeny. Over half of the progeny (58.3%) failed to amplify a paternal allele at one of seven loci; however, fewer than 5% were missing paternal genotypes at two or more loci. Null alleles accounted for a very small percentage of the missing data, while simple failure to amplify some primers in specific crosses appeared to be the cause in most cases. The failure to amplify a product was most acute on the maternal side (*P. deltoides*). For two markers (PMGC 2235 and PMGC 2675), five of the seven maternal parents displayed distorted allelic segregation due to a failure to amplify a product. For the remaining five

**Table 2** Properties of seven SSR loci selected for paternity studies in *P. deltoides* (*D*) by *P. nigra* (*N*) crosses (*P. nigra* is paternal parent)

| Locus | Species | *A* | *k* | *N* | $H_{(O)}$ | $H_{(E)}$ | Excl (1) | Excl (2) | Null freq | Use |
|---|---|---|---|---|---|---|---|---|---|---|
| PMGC 649 | T | 94–158 | 19 | 19 | 0.895 | 0.957 | 0.759 | 0.863 | 0.019 | 1 |
| | D | 100–152 | 17 | 17 | 0.765 | 0.959 | 0.753 | 0.859 | 0.098 | 1* |
| | N | 92–108 | 7 | 18 | 0.722 | 0.754 | 0.336 | 0.513 | 0.013 | 1 |
| PMGC 14 | T | 186–210 | 7 | 19 | 0.632 | 0.804 | 0.406 | 0.586 | 0.118 | 1 |
| | D | 188–191 | 2 | 19 | 0.579 | 0.508 | 0.122 | 0.186 | 0.079 | 2 |
| | N | 197–220 | 7 | 20 | 0.600 | 0.771 | 0.350 | 0.526 | 0.116 | 1 |
| PMGC 433 | T | 198–230 | 14 | 18 | 0.722 | 0.927 | 0.667 | 0.801 | 0.110 | 1 |
| | D | 193–221 | 15 | 19 | 0.684 | 0.909 | 0.630 | 0.774 | 0.118 | 1 |
| | N | 196–228 | 8 | 18 | 0.556 | 0.514 | 0.147 | 0.321 | 0.069 | 1 |
| PMGC 2221 | T | 67–138 | 21 | 18 | 0.667 | 0.959 | 0.759 | 0.863 | 0.167 | 1 |
| | D | 73–287 | 19 | 19 | 0.842 | 0.950 | 0.739 | 0.849 | 0.048 | 1 |
| | N | 81–136 | 15 | 19 | 0.526 | 0.937 | 0.700 | 0.824 | 0.265 | 1 |
| PMGC 2235 | T | 111–152 | 16 | 18 | 0.833 | 0.941 | 0.708 | 0.829 | 0.046 | 1 |
| | D | 114–161 | 15 | 18 | 0.611 | 0.927 | 0.670 | 0.803 | 0.190 | 1 |
| | N | 111–132 | 9 | 19 | 0.895 | 0.898 | 0.591 | 0.745 | 0.011 | 1 |
| PMGC 2675 | T | 165–232 | 14 | 17 | 0.353 | 0.922 | 0.656 | 0.793 | 0.441 | 2* |
| | D | 161–220 | 18 | 19 | 0.632 | 0.923 | 0.669 | 0.801 | 0.180 | 1 |
| | N | 152–208 | 10 | 17 | 0.412 | 0.863 | 0.521 | 0.688 | 0.347 | 1 |
| PMGC 2885 | T | 291–316 | 7 | 18 | 0.667 | 0.778 | 0.364 | 0.543 | 0.067 | 1 |
| | D | 297–316 | 10 | 19 | 0.789 | 0.839 | 0.483 | 0.657 | 0.013 | 1 |
| | N | 288–322 | 8 | 20 | 0.650 | 0.817 | 0.430 | 0.608 | 0.100 | 1 |
| AVERAGE OVER ALL SEVEN LOCI | T | | 14.0 | 18.1 | 0.681 | 0.898 | 0.617 | 0.754 | 0.138 | |
| | D | | 13.8 | 18.6 | 0.700 | 0.859 | 0.613 | 0.704 | 0.104 | |
| | N | | 9.3 | 18.7 | 0.623 | 0.793 | 0.439 | 0.604 | 0.132 | |

Values are given for *P. trichocarpa* as well. *A* Range of allele size in bp, *k* number of alleles, *N* number of individuals typed, $H_{(O)}$ observed heterozygosity, $H_{(E)}$ expected heterozygosity, *Excl(1)* average exclusion probability without information on one parent, *Excl(2)* average exclusion probability given information on known (female) parent, *Null freq* estimated null allele frequency, Use: 1 indicates likely useful locus, 2 indicates possibly useful locus

*Indicates issues with genetic interpretability

loci, only three of 35 cases displayed segregation distortion of maternal alleles ($p$=0.05, $\chi^2 \geq 3.84$, 1 *df*). Marker PMGC 2235 is known to occur on chromosome IV which is comprised almost entirely of maternally biased segregation ratios [17]. Interestingly, two of the 16 paternal parents (numbers 12 and 16) possessed triploid genotypes and a third parent (number 9), a tetraploid genotype, for marker PMGC 2675. Whether this was causal in the aforementioned amplification problem is unknown. To properly account for paternal parents with three or four alleles in the paternal assignment program, additional dummy genotypes were created for these three parents. It should be noted that polyploidy in poplars is not uncommon [3].

## Paternal reproductive success

The PFL program assigned unambiguous paternity to 86% (309) of the progeny based on a single mismatch (one mismatch excludes a parent) and 94.7% based on two mismatches. Of the remaining 19 progeny, 15 were assigned two potential paternal parents, three were assigned three paternal parents, and a single individual could not be matched. For numerical summary purposes, multiple paternal assignments were treated proportionately. That is, each of two potential parents received credit for 0.5 progeny, and each of three potential parents received credit for 0.33 progeny. The theoretical expectation of exclusion probability exceeding 99% was above the observed value of 94.7%. Several factors likely contributed to this shortfall: genotyping error, including missing data, progeny that were undifferentiated genetically (half-sibs), and/or pollen contamination. Indeed, five of the 15 cases sporting two potential pollen parents assigned the half-sib paternal parents number 7 and number 8. Contamination, though unlikely, in the controlled-cross conditions of this experiment, was still possible and may have accounted for the lone progeny that had no parental assignment.

All 16 paternal parents in the polymix were represented in the progeny pool, although representation was highly variable (Table 3). Clearly, the null hypothesis that all parents would be represented equally was rejected ($p$<0.001, $\chi^2$=219.1, 15 *df*). Over all seven females, the expectation is that each paternal parent would be represented by 22.3 progeny (3.2 per individual female). Actual paternal contribution varied from 2.5 to 62.0 (1.0 to 17.0%) across all female parents and ranged from 0–12% for individual female parents. The top eight paternal parents sired 83.3% of the progeny. In general, the top eight parents were well represented in each cross, though there were instances in which these males failed to sire a progeny for a given female (i.e., Male number 1, Cross H4). The reverse was also true, i.e., a number of parents that faired poorly overall did quite well with individual females. The expected sample size for each male by female combination was too low to consider performing interaction chi-square tests.

It is noteworthy that paternal success appeared to be unrelated to pollen vigor, as measured by proportion of pollen grains germinating in a short term test ($r^2$=0.057). Indeed, the least represented pollen parent (number 8) had the highest pollen germination score (>25%). Still, reproductive success was clearly low for all parents with germination scores of one. Given the observation that a few single parent controlled crosses with pollen parents that had germination scores of one resulted in no filled seed, it is surprising to see any representation in the polymix progeny for these parents. One explanation could be the mentor pollen effect [14]. The mentor pollen technique has

**Table 3** Number of progeny assigned to each of 16 paternal parents in a polymix for each of seven crosses (H2–H9), the total number and proportion of progeny assigned each parent, and a measure of pollen viability based on germination percentage (<10%=1, 10–15%=2, 15–20%=3, and 20–25%=4)

| | Female (*P. deltoides*) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Male (*P. nigra*) | H2 | H3 | H4 | H5 | H6 | H8 | H9 | Total | Proportion of total | Pollen viability |
| 1 | 2.0 | 5.5 | 0.0 | 8.0 | 8.0 | 4.5 | 4.5 | 32.5 | 0.09 | 2 |
| 2 | 1.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 2.0 | 3.3 | 0.01 | 1 |
| 3 | 9.0 | 7.5 | 6.0 | 5.0 | 4.0 | 5.0 | 4.0 | 40.5 | 0.11 | 3 |
| 4 | 2.3 | 4.5 | 4.0 | 4.0 | 7.8 | 10.0 | 3.5 | 36.1 | 0.10 | 4 |
| 5 | 4.0 | 3.0 | 8.0 | 4.0 | 1.3 | 6.0 | 10.0 | 36.3 | 0.10 | 4 |
| 6 | 12.0 | 7.0 | 11.0 | 7.0 | 8.5 | 11.0 | 5.5 | 62.0 | 0.17 | 2 |
| 7 | 0.8 | 1.0 | 1.5 | 1.5 | 0.0 | 0.0 | 3.0 | 7.8 | 0.02 | 1 |
| 8 | 0.5 | 0.0 | 0.5 | 0.5 | 0.0 | 0.0 | 1.0 | 2.5 | 0.01 | 4 |
| 9 | 5.3 | 1.0 | 2.0 | 6.5 | 1.0 | 0.5 | 2.5 | 18.8 | 0.05 | 4 |
| 10 | 7.0 | 11.0 | 5.0 | 5.0 | 10.0 | 3.0 | 6.0 | 47.0 | 0.13 | 2 |
| 11 | 0.0 | 2.5 | 0.0 | 0.5 | 1.5 | 2.0 | 4.0 | 10.5 | 0.03 | 1 |
| 12 | 2.0 | 2.0 | 0.0 | 5.0 | 2.0 | 1.0 | 2.0 | 14.0 | 0.04 | 3 |
| 13 | 1.0 | 2.0 | 2.0 | 1.0 | 1.3 | 1.0 | 0.0 | 8.3 | 0.02 | 4 |
| 14 | 2.0 | 2.0 | 7.0 | 0.0 | 3.3 | 7.0 | 3.0 | 24.3 | 0.07 | 2 |
| 15 | 1.0 | 0.0 | 1.0 | 1.0 | 2.8 | 0.0 | 1.0 | 6.8 | 0.02 | 1 |
| 16 | 0.0 | 0.0 | 4.0 | 0.0 | 0.0 | 1.3 | 1.0 | 6.3 | 0.02 | 4 |
| Totals | 50.0 | 49.0 | 52.0 | 49.0 | 52.0 | 52.0 | 53.0 | 357.0 | 1.00 | |

been used to make inter-specific crosses that otherwise cannot be made. The desired pollen is mixed with irradiated (killed) compatible pollen before pollination and fertilization is facilitated. The combination of pollens in this case may have actually aided pollen lots of low viability.

Finally, all three paternal parents that possessed polyploid genotypes for marker PMGC 2675 were underrepresented in the progeny pool (mean of 13 progeny per parent, overall) even though they all possessed high pollen germination scores.

Though the experimental design and statistical rigor of the experiment are insufficient to differentiate among all the possible reasons for unequal paternal success, the results do guide speculation. The lack of association between vigor and paternal success implies pre-zygotic competition based on number of pollen grains competing is not a critical factor although issues such as pollen tube growth rate may still be relevant (assuming rate of growth is unrelated to germination success). Post-zygotic competition in the form of genetic complementation between male and female parents could explain much of the pattern of paternal success observed. This may be particularly relevant in this case where inter-specific crosses were made. The correspondence between DRS and paternal half-sibs among the pollen parents appears to support this hypothesis. For instance paternal half-sib pairs number 7 and number 8, and number 2 and number 15 each exhibited very poor reproductive success for both individuals (mean of 1.5% in each case), while each of the three pollen parents in the half-sib that included number 5, number 12, and number 14 contributed significantly (mean of 7.0% per parent) to the progeny pool.

## Applicability of PMX/WPA for hybrid poplar breeding

PMX/WPA was conceived for recurrent selection breeding programs in which pedigree control is vital to reduce or eliminate inbreeding in advanced generations while simultaneously improving gain and decreasing breeding costs. For inter-specific $F_1$ hybrid breeding programs, such as the one described here, pedigree control for inbreeding is not directly relevant, though knowing the parentage of individual progeny is still valuable. Managing a pedigree against inbreeding would be relevant if reciprocal recurrent selection programs exist for each parental species to identify the best parents to enter the hybrid program. More importantly, PMX/WPA offers a method for increasing the number of parental combinations created, ostensibly improving the chances of finding the best combination at a reasonable cost. This is particularly true if the chosen alternative approach is a disconnected factorial design. A potential tradeoff, relative to traditional factorial designs, is that fewer progeny per cross are created with PMX/WPA, assuming that progeny test size remains the same for the two designs. It is necessary to understand the genetic variance structure of "among parents" vs "within parents" to determine if this trade off is significant. While it is too early to determine if gain was improved in this study, it is possible to evaluate the costs of deploying PMX/WPA relative to the traditional factorial mating design used in the hybrid poplar breeding in this program. For the combination of activities that included grafting of breeding materials, pollen handling, making controlled crosses, and seed sowing, the polymix approach cost was ~30% of the traditional factorial approach ($9,716.00 vs $31,556.00). The added costs of genotyping all parents and a few selected progeny would be nominal (<$500.00), making this approach economically quite attractive in an operational setting.

## Summary

Significant logistical and economic incentives exist for adopting PMX/WPA for the creation of hybrid poplars in the program described here. Though not particularly surprising, the skewed paternal contribution to the progeny pool is noteworthy and suggests follow-up studies to determine if pollen competition induced by polymix breeding prevents otherwise good crosses from occurring while simultaneously encouraging fertilization from pollen sources with low vigor. Of the 112 potential crosses in this polymix study, 92 were represented in the progeny pool. This contrasts with the 49 crosses that would have occurred under the factorial study. Even the least successful pollen parent could be represented by ~70 progeny in the operational field tests (0.7% success rate in sample × 9,800 progeny in test). Still, it would be desirable to have the remaining 20 potential crosses represented if the highest possible predicted gains are to be achieved.

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ORIGINAL PAPER

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# Retrospective selection of elite parent trees using paternity testing with microsatellite markers: an alternative short term breeding tactic for *Eucalyptus*

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**Abstract** The conventional way to drive modifications in old forest tree seed orchards is to establish progeny trials involving each parent tree and then evaluate its contribution to the performance of the progeny by estimating its general and specific combining ability (GCA and SCA). In this work, we successfully applied an alternative parent selection tactic based on paternity testing of superior offspring derived from a hybrid seed orchard established with a single *Eucalyptus grandis* seed parents and six *E. urophylla* pollen parents. A battery of 14 microsatellite markers was used to carry out parentage tests of 256 progeny individuals including two independent samples of selected trees and one control unselected sample, all derived from 6-year-old forest stands in eastern Brazil. Paternity determination was carried out for all progeny individuals by a sequential paternity exclusion procedure. Exclusion was declared only when the obligatory paternal allele in the progeny tree was not present in the alleged parent tree for at least four independent markers to avoid false exclusions due to mutation or null alleles. After maternity checks to identify seed mixtures and selfed individuals, the paternity tests revealed that approximately 29% of the offspring was sired by pollen parents outside the orchard. No selfed progeny were found in the selected samples. Three pollen parents were found to have sired essentially all of the offspring in the samples of selected and non-selected progeny individuals. One of these three parents sired significantly more selected progeny than unselected ones ($P \leq 0.0002$ in a Fisher exact test). Based on these results, low-reproductive-successful parents were culled from the orchard, and management procedures were adopted to minimize external pollen contamination. A significant difference ($P<0.01$) in mean annual increment was observed between forest stands produced with seed from the orchard before and after selection of parents and revitalization of the orchard. An average realized gain of 24.3% in volume growth was obtained from the selection of parents as measured in forest stands at age 2–4 years. The marker-assisted tree-breeding tactic presented herein efficiently identified top parents in a seed orchard and resulted in an improved seed variety. It should be applicable for rapidly improving the output quality of seed orchards, especially when an emergency demand for improved seed is faced by the breeder.

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## Introduction

Eucalypts are the most widely planted hardwood trees in the world. Estimates made in 2000 (FAO 2000) indicated that the eucalypt plantation area was globally greater than 17.8 million hectares, with India being the largest planter with 8.0 million hectares—mostly in extensive low-productivity plantations—followed by Brazil with 3.0 million hectares of mostly intensively cultivated clonal plantations of industrial forests reaching average productivities of 45–60 $m^3$/ha per year (Mora and Garcia 2000). Elite hybrid clones consisting of *Eucalyptus grandis* and *E. urophylla* are extensively used by the cellulose and paper industry because of its wood quality, rapid growth and high volumetric yield (Bertolucci et al. 1995).

In Brazil, eucalypt genetic improvement programs were initiated in the early 1970s. The main purposes of the breeding programs were to match the most adapted

species to the existing environmental conditions and to provide sufficient plant stock of reasonable genetic quality (Brune and Zobel 1981). Several *E. grandis*, *E. urophylla* and *E. saligna* seed orchards were established on the basis of initial results of available provenance/progeny trials. Furthermore, due to the early observation of hybrid superiority for growth coupled to resistance to eucalypt canker caused by *Cryphonectria cubensis* (Bruner) Hodges, hybrid seed orchards of *E. grandis* × *E. urophylla*, were also established, thereby generating the so-called Urograndis seed variety (Campinhos 1980; Vigneron 1991; Eldridge et al. 1993). These open-pollinated seed orchards often involved the deployment of one or a few self-incompatible or male-sterile *E. grandis* trees as female parents and a number of selected *E. urophylla* as male parents to ensure enough pollen pressure (Bertolucci et al. 1995).

Clonal propagation of elite trees started in the 1980s followed the initial breeding/testing/selection efforts. This new technology, involving the establishment of true clonal stands through vegetative propagation via rooted cuttings, was developed and applied on a wide scale, thereby providing significant productivity and uniformity gains (Campinhos 1980; Brandão et al. 1984) and consequently reducing the demand for seedlings in the establishment of new forests. However, recent increases in the industrial demand for wood have forced forest companies to rapidly expand planting areas and establish partnership programs with farmers. The only way to fulfill this immediate challenge has been to use seeds from first-generation breeding orchards to complement the production of rooted cuttings. The development of improved seed varieties has thus become necessary again in the context of current breeding programs, both by establishing new orchards with trees of high general and specific combining ability (GCA and SCA, respectively) and by restructuring old ones.

The conventional way to drive modifications in old seed orchards is to establish progeny trials to evaluate the contribution of the parent to the performance of its progeny by SCA estimation. This approach requires a long time to achieve its purpose (Namkoong et al. 1988). An alternative way to estimate the superiority of the parent trees of a seed orchard could be attained by a marker-assisted breeding tactic: carrying out DNA-based paternity tests of superior progeny trees in forest stands established with open-pollinated seeds from the orchard. Parents that display a low frequency of superior offspring could be culled from the orchard, thus practicing a backwards selection that would result in an improved seed variety involving exclusively trees of higher GCA and SCA (Ribeiro et al. 1998; Grattapaglia 2000).

A similar approach, termed PMX/WPA (polymix breeding with parental analysis) was evaluated as an alternative solution to full-sib crosses in pine breeding programs (Lambeth et al. 2001). As pointed out by those authors, although PMX breeding is easy, provides good estimates of breeding values and allows the testing of a larger number of parental combinations, pedigree control is lost. Instead of using a single pollen for each cross, the concept then proposed by Lambeth et al. (2001) was to use PMX breeding involving many male parents followed by paternity analysis of progeny with microsatellites, thus allowing full pedigree control.

Similar to the situation in humans (Hammond et al. 1994) and domestic animals (Glowatzki-Mullis et al. 1995), the high degree of multi-allelism and the clear and simple codominant Mendelian inheritance of microsatellites provide an extremely powerful system for the unique identification of *Eucalyptus* individuals in parentage testing, particularly when individuals are expected to be related (Brondani et al. 1998). In the study reported here, we applied a marker-assisted breeding tactic to an old *E. grandis* × *E. urophylla* seed orchard with the specific objectives of verifying the differential reproductive success of six male parents and potentially identifying those with higher SCA with the maternal tree to develop an improved seed variety.

## Materials and methods

### Seed orchard

The seed orchard studied was established in 1982 in Aracruz (latitude 19°49", longitude 40°16") in Espirito Santo State, Brazil by Aracruz Celulose S.A. Six *Eucalyptus urophylla* plus trees identified as being canker-free were selected from provenance/progeny trials of open-pollinated, half-sib families originally collected on the Bessi-Lau, Flores and Timor Islands in Indonesia. These six trees were clonally propagated by grafting and constituted the male parents in the seed orchard. A single *E. grandis* plus tree selected from a progeny trial of open-pollinated, half-sib families originally collected in Atherton (Australia) was used as the female parent. This particular female parent was selected due to its good growth performance and putative self-incompatibility that was deduced from its very low seed set in controlled self-pollinations (Bertolucci et al. 1995). The seven parent trees were clonally replicated and planted in a 6×6 spacing in 267 hexagonal plots totaling 7.5 ha, with each of the *E. grandis* maternal tree surrounded by the six pollinator trees. The orchard was surrounded by a 300-m wide belt of native tropical forest to provide isolation from any pollen coming from nearby eucalypt plantations. Seeds were only collected from the *E. grandis* maternal tree.

### Selection of progeny individuals

Sampling of progeny individuals was carried out in 6-year old stands established with seeds derived from the seed orchard studied. Two independent samples of 72 selected trees each were taken from two different forested areas—SSA selected sample A and SSB selected sample B. Individual trees with a circumference at breast height (CBH) above one standard deviation from the mean (mean = 55±15 cm) were selected, reaching a selection intensity of approximately 1:200. A control sample of 72 random non-selected (RNS) trees was also taken to allow a comparative analysis with the selected tree samples. To verify potential trees derived from selfing of the maternal tree, we also investigated a sample of ten trees with stunted growth and a sample of 30 trees that displayed symptoms of chlorotic leaves. Total genomic DNA was extracted from adult leaf tissue following the protocol described by Grattapaglia and Sederoff (1994).

### Microsatellite genotyping

Forty-seven microsatellite markers developed by Brondani et al. (1998, 2002) were screened for polymorphisms among the seven parent trees in order to identify a battery of markers with higher information content for the proposed study. Microsatellite marker amplification and detection were performed as described earlier (Brondani et al. 1998). The amplified products were separated on 4% denaturing polyacrylamide gels, stained with silver nitrate (Bassam et al. 1991) and sized by comparison to a 10-bp DNA ladder standard (GibcoBRL, Gaithersburg, Md.) on a computer screen. Allele sizes were estimated using the software SEQAID II (Rhoads and Roufa 1990), taking into consideration the expected allelic series in basepairs for the locus. Multiplex loading in the same gel was carried out for up to three microsatellite loci simultaneously.

### Paternity testing

Gels were scanned, and scoring was carried out manually on a computer screen. Multi-locus genotypes were determined for all seven parent trees and the five samples of progeny individuals (SSA, SSB, RNS, stunted and chlorotic). Maternity of the *E. grandis* tree was first checked, and seed contaminants identified. Paternity determination was carried out for all progeny individuals by a sequential paternity exclusion procedure. Exclusion was declared only when the obligatory paternal allele in the progeny tree was not present in the alleged parent tree for at least four independent markers to avoid false exclusions due to mutation in the microsatellite marker. Paternity was ultimately declared when the alleged parent tree shared the obligatory paternal allele at all of the microsatellite markers tested. Because the number of potential pollen parents was limited, paternity declaration was deterministic and did not involve the calculation of a paternity index using likelihood-ratio methods. Progeny that were not assigned to a specific pollen parent were classified either as selfs or as having been derived from outside pollen contamination.

### Paternity in selected versus non-selected trees

For each pollen parent tree that effectively sired progeny individuals, a two-tailed Fisher exact probability test (Fisher 1934) was used to test the null hypothesis that the proportion of sired progeny individuals is not significantly different between the selected and non selected offspring samples.

### Improvement of productivity with parental selection

The male parents displaying the lowest reproductive success were eliminated from the orchard in the aim of producing a new improved seed variety. Realized gain in mean annual increment (MAI) ($m^3$/ha per year) was evaluated by means of a comparative inventory between stands established with seedlings derived from the original orchard (78 stands) and seedlings derived from the improved orchard following the culling of the worst parents (18 stands). These forest stands were 2–4 years old and had an average size of 20 ha with 1,111 trees per hectare; they were located at uniform sites in Bahia State. Student's *t*-probability test was used to test for significant differences in MAI between the old and the new variety.

## Results

Out of the 47 microsatellite markers screened, a battery of 14 markers was ultimately selected and used throughout the study (Table 1). On the basis of multi-locus microsatellite data, paternity testing tables were set up for each progeny individual, and a sequential exclusion procedure was applied (Table 2). In each progeny population sample, the analysis of 14 microsatellite markers allowed immediate identification of: (1) progeny from other seed sources (*E. grandis* mother excluded); (2) progeny derived from outside pollen contamination (all six fathers excluded); (3) selfed individuals (only maternal alleles); (4) progeny that belonged to one of the six pollen parent (Table 3). Approximately 29% of the offspring was sired by pollen parents located outside of the orchard, indicating the ineffectiveness of the seed orchard isolation zone. No selfed progeny were found in the selected samples, which confirmed self-incompatibility of the seed donor parent. However, 6 of the 72 randomly taken trees (8.3%) and eight of the ten stunted trees (80%) were selfs. The sample of chlorotic trees analyzed indicated that these were sired either by outside pollen (Table 3) or by pollen tree no. 6 (Table 4).

Pollen parent trees nos. 3 and 5 displayed indistinguishable multi-locus genotypes at all 14 microsatellite markers (data not shown). Analyses were repeated three

**Table 1** Microsatellite markers used in the paternity testing of progeny individuals. The linkage group is as determined in Brondani et al. (2002) (*nd* not determined)

| Microsatellite marker | Forward primer 5′–3′ | Reverse primer 5′–3′ | Observed heterozygosity | Number of alleles | Linkage group |
|---|---|---|---|---|---|
| Embra 6 | AgAgAATTgCTCTTCATggA | gAAAAgTCTgCAAAgTCTgC | 0.50 | 5 | 1 |
| Embra 10 | gTAAAgACATAgTgAAgACATTCC | AgACAgTACgTTCTCTAgCTCA | 0.83 | 7 | 10 |
| Embra 11 | gCTTAgAATTTgCCTAAACC | gTAAAATCCATgggCAAg | 0.17 | 4 | 1 |
| Embra 16 | CAACgTTCCCCTTTCTTC | ATgTTAggCCAAACCCAg | 0.67 | 7 | 1 |
| Embra 21 | ACAAgggAAACTTgATCg | ggAACCgAACATAgCAAg | 1.00 | 7 | 10 |
| Embra 22 | gCACATgCACACACggTTg | AAggCCAgTggTCgTgAgTC | 0.67 | 7 | 11 |
| Embra 27 | ATAACCACACCAATCTgCA | TATAgCTCgAACgCTCAAC | 1.00 | 6 | 2 |
| Embra 30 | TTAgTTgAATCCAACCATTg | TATATAAggTgCAAATAATAAA-Cg | 1.00 | 7 | 8 |
| Embra 37 | CACCTCTCCAAACTACACAA | CTCCTCTCTCTTCACCATTC | 0.83 | 9 | 5 |
| Embra 40 | AAAgTATCTCCACgCTTCAT | TCCCAATCATgATCTTCAg | 1.00 | 9 | 10 |
| Embra 49 | ATTATTggTTCATATTgAAAACC | AgATAgAgATTgAgTgAgACCC | 0.67 | 9 | 3 |
| Embra 52 | TAATCAgCATTAgCgAAAgA | CgTATATgTTCAgAgTCAATCC | 0.67 | 9 | 7 |
| Embra 53 | ATTAgCTTTTCTgTAACCCg | gAATggACAAgCTCTgATg | 1.00 | 7 | 8 |
| Embra 131 | ACTTAACATCTATACATATTTg | TgTCCTATCTggCTCA | 1.00 | 6 | nd |

**Table 2** Example of paternity testing of a selected progeny individual with six microsatellite markers. Alleles were numbered from the smallest to the largest sized allele. At each locus, the maternal allele is indicated in italic and the obligatory paternal allele is indicated in bold and underlined. Pollen parent tree no. 6 was declared the father of individual 46

| EMBRA locus | Mother tree | Progeny individual 46 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|---|
| 16 | *4/5* | **2**/*5* | 1/3 | 1/1 | 1/2 | 4/4 | **2**/4 | **2**/3 |
| 37 | *1/2* | **2**/*6* | 7/7 | **6**/9 | 5/8 | 8/8 | **7**/8 | **6**/6 |
| 6 | *3/5* | **1**/*3* | 3/5 | **4**/4 | 4/4 | 3/4 | 4/4 | **1**/4 |
| 131 | *4/4* | **2**/*4* | 1/6 | 1/6 | 1/5 | 3/6 | 1/5 | **2**/5 |
| 27 | *1/3* | **3**/*6* | 2/5 | 2/4 | 2/4 | 5/5 | 4/4 | **4**/6 |
| 10 | *3/5* | *5*/**7** | 5/5 | 2/5 | 1/5 | 5/5 | 1/4 | 5/**7** |

**Table 3** Parentage determination in the five progeny samples

| Progeny sample | Number of individuals | Non-maternity (seed mixture) | Sired by outside pollen parents | Selfed | Sired by orchard pollen parents |
|---|---|---|---|---|---|
| Selected-plus trees (SSA) | 72 | 1 | 17 | 0 | 54 |
| Selected-plus trees (SSB) | 72 | 4 | 23 | 0 | 45 |
| Non-selected trees (RNS) | 72 | 14 | 22 | 6 | 30 |
| Chlorotic trees | 30 | 1 | 11 | 0 | 18 |
| Stunted trees | 10 | 0 | 0 | 8 | 2 |
| Total | 256 | 20 | 73 | 14 | 149 |

**Table 4** Number of offspring sired by each pollen parent in the progeny samples from the seed orchard

| Progeny sample | Pollen parents | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5[a] | 6 |
| SSA—Selected sample A | 32 | 11 | 0 | 0 | - | 11 |
| SSB—Selected sample B | 29 | 1 | 1 | 0 | - | 14 |
| Non-selected trees | 5 | 8 | 0 | 0 | - | 17 |
| Chlorotic trees | 0 | 0 | 0 | 0 | - | 18 |
| Stunted trees | 0 | 2 | 0 | 0 | - | 0 |
| Total | 66 | 22 | 1 | 0 | - | 60 |

[a] Pollen parents nos. 3 and 5 are the same tree

times with leaf samples collected from different clonal ramets; these confirmed the results. A probability of a random match between trees nos. 3 and 5 was estimated to be 1 in 89 billion using allele frequency estimates for these loci for *E. grandis* and *E. urophylla* (R. Brondani and M. Kirst, unpublished results).

Pollen parents nos. 1, 2 and 6 sired 148 out of the 149 offspring sired with orchard pollen. Pollen parents nos. 3 and 5, actually the same tree, sired only a single offspring in SSB, and parent no. 4 did not sire any offspring (Table 4). For parents nos. 1 and 6, significant differences were found in the proportions of progeny individuals in both selected samples versus the non-selected sample of trees. However, while pollen tree no. 1 sired significantly more selected progeny than non-selected ones in both SSA and SSB ($P$=0.0002 and 0.00005 respectively), pollen tree no. 6 sired significantly more offspring in the non-selected tree sample for both comparisons ($P$= 0.00141 and 0.03365, respectively) (Table 5). For pollen tree no. 2, no significant difference was observed between the proportions of sired offspring in SSA versus RNS ($P$= 0.58942), but significantly more RNS offspring were sired as compared to SSB ($P$=0.00221) (Table 5). A similar trend was observed when the test was carried out analyzing the outside pollen contribution—i.e. either the outside pollen contribution sired more non-selected offspring in SSA (22 vs. 17, Table 3) ($P$=0.00028), or no difference was detected in SSB ($P$=0.09132) (Table 5).

For practical breeding purposes, since parents nos. 1, 2 and 6 displayed significant reproductive success and effectively contributed to the generation of superior hybrid trees, they were kept in the orchard. Parents nos. 3, 4 and 5 did not contribute to the generation of superior offspring and were thus eliminated. A significant difference (P 0.01) in volume growth was observed between forest stands produced from orchard seeds before and after culling the worst parents (Table 6). The new seed variety (MAI = 44.5±5.8 $m^3$/ha per year) resulted in forest stands that were on average 24.3% more productive in volume growth than the original seed variety (MAI = 35.8±2.3 $m^3$/ha per year).

**Table 5** Results ($P$-values) of Fisher exact contingency tests for binomial proportions of sired progeny individuals in the selected and non-selected offspring samples for the different pollen sources

| Pollen source | SSA—Selected sample A | SSB—Selected sample B |
|---|---|---|
| Pollen tree no. 1 | 0.00020 | 0.00005 |
| Pollen tree no. 2 | 0.58942 | 0.00221 |
| Pollen tree no. 6 | 0.00141 | 0.03365 |
| Outside pollen | 0.00028 | 0.09132 |

**Table 6** Number of commercial stands (average 20 ha, with 1,111 trees/ha) submitted to inventory. Mean annual increment (MAI) in cubic meters per hectare per year is given as the mean and standard deviation for seed varieties produced before and after elimination of parents with a low reproductive success for revitalization of the orchard

| Seed variety | Number of stands | MAI (mean) | MAI (standard deviation) |
|---|---|---|---|
| Old (before culling) | 78 | 35.8 | 2.3 |
| New (after culling) | 18 | 44.5 | 5.8 |

## Discussion

A battery of 14 polymorphic markers was selected to carry out parentage tests of 256 progeny individuals. Maternity checks were carried on all individuals and, following the exclusion of seed mixtures and selfed individuals, paternity tests were carried out. Markers were specifically selected to allow easy scoring in polyacrylamide gels and to maximize allelic differences between the group of five pollen parents and between them and the maternal parent. This was possible as the five pollen parents were unrelated and the maternal parent belonged to a different species. We also specifically looked for markers for which all of the parents, maternal and pollen parents, were heterozygous so as to avoid the occurrence of null alleles that could result in false paternity exclusions (Moller 1995). A relatively large number of alleles were observed for the microsatellite markers used in the study, and for 6 out of the 14 markers all the parents were heterozygous, thereby allowing a significant discrimination power and confidence in the parentage testing (Table 1).

We also were interested in markers for which the maternal parent did not share alleles with any of the six pollen parents. Out of the 14 markers, three had this configuration. Based on these three markers the analysis of maternity versus non-maternity—i.e. seed mixture—was very fast and efficient. Progeny individuals that did not have any of the two maternal alleles were declared to result from the seed mixture. These loci were also very useful for determining the presence of selfed progeny individuals. The presence of homozygous loci in the maternal tree would also be efficient for the maternity checks, however null alleles could lead to false maternity exclusions.

The analysis of pollen contamination in the orchard was based on multiple paternity exclusions when the six pollen parents were tested. In this procedure it was essential to have genotypes for several loci to declare non-paternity with absolute confidence with a minimum of three excluding loci to avoid false exclusions due to mutations (Gunn et al. 1997). Di-nucleotide repeat microsatellites have been reported to have higher mutation rates following the step-wise mutation model (Valdes 1993) than longer repeat motifs in humans (Brinkmann et al. 1999) and, more recently, in maize (Vigouroux et al. 2002). Because no data are available yet on the frequency of null alleles for *Eucalyptus* microsatellites and, in fact, for the vast majority of plant species, our premise was that such a behavior would be the case for *Eucalyptus* as well. Although the most informative six markers for which all trees were heterozygous could be sufficient, we used an over-abundance of marker loci in the paternity testing to avoid false exclusions due to the inheritance of mutated alleles from the pollen parent to progeny individuals.

When we excluded the offspring resulting from pollen contamination and took into consideration the fact that pollen trees nos. 3 and 5 were clonal (i.e. identical), a total of 149 progeny individuals were tested against the five unique pollen parents, making a total of 745 paternity tests. In these 745 paternity tests, a putative occurrence of a null allele was observed at locus EMBRA6 where pollen parent no. 1 was apparently homozygous. However, 28 out of a total of 66 offspring lacked the obligatory paternal allele. At all other loci offspring and pollen parent shared the paternal allele correctly. This result strongly suggests the occurrence of a null allele in pollen parent no. 1 that, as expected, was transmitted to approximately half (28/66=42%) of its progeny. This locus was not considered in the paternity analysis for pollen tree no. 1.

A total of 745×14= 10,430 allelic transmission analyses were carried out in the paternity testing. There were five putative occurrences of paternal mutations—i.e. where the obligatory paternal allele was different, usually by a single dinucleotide step, from the allele observed in the pollen parent. Again, at all other loci, a full consistency of obligatory paternal allele was observed. The resulting mutation frequency estimate of $4.8\times10^{-4}$ is very similar to recent estimates in maize where the mutation rate per generation was estimated to be $7.7\times10^{-4}$ for microsatellites with dinucleotide repeat motifs (Vigouroux et al. 2002).

When the inconsistencies due to mutations and null alleles were not counted, the sequential procedure of paternity exclusion proved to be a very efficient and accurate method to quickly exclude non-fathers at several marker loci. By this approach, once a pollen parent was excluded in at least three loci, the remaining paternity tests were only carried out with the non-excluded fathers. Typically, however, all non-fathers were excluded in the analysis of the first five more informative loci. The proposed approach is therefore very efficient and should not demand the analysis of a large number of loci. It is obvious that as the number of mother trees and pollen parents increases and the relatedness of these trees also increases, confident paternity testing will require an increased number of polymorphic loci to reach conclusions as multiple males are often found to be genetically compatible with each offspring tested, even when the probability of excluding an unrelated male is high. In such

complex parentage testing situations, the deterministic approach used in this study is usually not possible, and likelihood based paternity inference methods become necessary (Meagher 1986; Marshall et al. 1998; Chaix et al. 2003).

A significant proportion of the analyzed offspring, approximately 29%, was sired by pollen parents located outside of the orchard. Campinhos et al. (1998) estimated a lower contamination rate of 14% in the same orchard based on isozyme markers, possibly due to limited informative polymorphism that would allow correct parental discrimination or simply due to a sampling effect. Outside pollinator trees did not contribute significantly to the generation of superior offspring individuals (Table 5), although one cannot preclude the possibility of some occasional superior trees deriving from pollen outside the orchard. Heavy pollen contamination has been observed in a number of studies with wind-pollinated conifer species (El-Kassaby and Ritland 1986; Harju and Nikkanen 1996; Pakkanen et al. 2000; Moriguchi et al. 2002), typically reducing the expected gains from seed orchards. For eucalypts, a number of studies have estimated the preferential mating system in natural populations and seed orchards using isozymes (e.g. Moran et al. 1989) and, recently, a complex pattern of mating was described in an *E. regnans* seed orchard in Australia. Gene dispersal was influenced by crop fecundity and orchard position of the mother trees with approximately 50% of effective pollen gametes coming from males more than 40 m away from mother trees, indicating that insect pollinators are efficient promoters of cross-fertilization (Burczyk et al. 2002). Furthermore, in a recent outcrossing rate study carried out in an *E. grandis* orchard in Madagascar, a pollination rate from outside the seed orchard of 39.2% was estimated based on six microsatellite markers (Chaix et al. 2003). The results from our study also indicate that in our exotic conditions the 300-m-wide belt of native tropical forest maintained around the orchard to provide genetic isolation has not been effective in preventing insect-mediated gene flow from nearby eucalypt plantations.

Pollen parents nos. 1, 2 and 6 displayed the highest male reproductive success, siring 148 out of the 149 offspring analyzed. All three pollen parents were thus maintained in the seed orchard. Pollen trees nos. 3, 5 and 4 did not father any offspring and were subsequently excluded. The absence of offspring from these parents is probably due to asynchronous flowering in relation to the maternal tree as well as temporal variation in the flower crop, suggesting that physical proximity between eucalypt tree crowns not necessarily implies successful mating in a seed orchard. Sampling effects may also have contributed to a certain extent. Differential male reproductive success was also observed in a recent *E. grandis* seed orchard study, where only 199 out of 349 potential male trees in the seed orchard contributed to the pollination of 440 offspring and at a very variable siring rate (Chaix et al. 2003).

Pollen tree no. 1 sired significantly more selected progeny than non-selected ones in both samples. For the other two pollen trees, either no significant difference was observed between the proportions of sired offspring in the selected versus non-selected samples or, in the case of pollen tree no. 6, significantly more non-selected trees were sired (Table 5). These results indicate a higher SCA and flowering synchrony of pollen tree no. 1 with the maternal tree, ensuing the immediate establishment of a bi-clonal seed orchard composed exclusively of pollen tree no. 1 and the *E. grandis* maternal tree.

Although a bi-clonal seed orchard could have been established, involving only pollen tree no. 1 and the *E. grandis* seed parent, due to spatial distribution of the remaining pollen donors and seed parent ramets and with the objective of maintaining a slightly larger effective population size, it was decided to leave all three pollen parents and to cull only those that clearly did not contribute to the generation of offspring. It seems therefore unreasonable to have accrued a 24% gain in volume growth leaving the same pollen donors, i.e. nos. 1, 2 and 6. However, besides culling the worst parents, a significant improvement was made in the silvicultural management of the orchard so as to minimize external pollen contribution and to improve flowering of the remaining pollen donors. Intensive fertilization regimes were applied to the orchard so as to stimulate abundant flowering, and a number of bee cages were introduced in the seed orchard so as to keep the bees from flying longer distances to harvest pollen and nectar. These measures were likely successful in minimizing external pollen contribution and at the same time increasing pollen contribution from the pollen donors in the orchard. Besides the revitalization of the orchard, it was also noted that the number of grafted ramets of pollen donors nos. 1, 2 and 6 was not balanced due to tree death since the time that the seeds used to establish the commercial plantation studied were harvested. The census number of ramets of pollen donor no. 1 was slightly larger that of pollen donor nos. 2 and 6, possibly due to its more adapted growth and vigor. A potentially larger contribution of pollen parent no. 1 to the new seed crop from the orchard also contributed to the increased volume growth.

As expected, no selfed progeny individuals were found among the selected trees. However, selfs were found in the random non-selected trees at a rate of 8.3%, which is in agreement with the selfing rates typically estimated in eucalypts (Griffin and Cotterill 1988; Gaiotto et al. 1997). This same selfing rate should therefore be expected even in a biclonal seed orchard. Not surprisingly, however, 80% of the stunted trees were selfs, thereby confirming the inbreeding depression effect on growth typically observed in controlled selfing experiments in eucalypts (Griffin and Cotterill 1988; Hardner and Potts 1995). Finally, no selfs were found in the sample of chlorotic trees. The paternity testing results suggest a specific association between pollen parent no. 6 and the appearance of leaf chlorosis, although 11 out of the 30 chlorotic trees were sired by outside pollen parents. The establish-

ment of a more efficient barrier to outside pollen contamination should minimize the manifestation of this symptom.

Our study demonstrates that a marker-assisted breeding tactic involving parentage testing with microsatellite markers can efficiently and rapidly be applied to existing seed orchards with the specific objectives of verifying the differential male reproductive success of pollen parents and identifying those that successfully generate superior offspring. It should be pointed out, however, that the tactic applied in this study represents an alternative measure in situations where time is the critical issue, the breeder is faced with an emergency demand for improved seeds from existing orchards and no data are available on progeny testing of trees but rather only on commercial plantations derived from the seed orchard. This tactic is certainly no substitute for a well-conducted progeny trial followed by estimation of parental breeding values for backwards selection. We have shown, however, that this retrospective selection procedure coupled to improved management of the seed orchard did in fact result in an improved seed variety displaying a significant gain in volume growth over the average performance of the seed lots derived from the original unselected orchard.

This short-term marker-assisted breeding tactic should be applicable to the improvement of old seed orchards currently supplying planting stock of other forest tree species. Although this demonstration experiment was carried out on a relatively small sample of parent trees, given an adequate screening and selection of a battery of informative microsatellite markers, sufficient power of discrimination can easily be attained to resolve paternity and maternity of offspring derived from orchards consisting of several tens of unrelated parents. Relatedness among the orchard parents could be a problem when a limited number of markers are available. Lambeth et al. (2001), after genotyping a set of 45 parental trees at seven chloroplast and three nuclear microsatellite loci, found that more markers would be necessary for unambiguous paternal determinations of progeny from a complete pollen mix due largely to relatedness in the pine population studied.

Finally, this tactic, as opposed to the conventional approach of estimating predicted gain based on controlled pollination and progeny trials, measures realized gain in an operational setting, which is a function not only of the SCA of pairs of parents but also of the effective reproductive success of mating between them, which is often a much more critical variable when large supplies of improved seeds are desired.

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## Population Management in the Age of Genomics

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Abstract: Continued population improvement will remain the foundation for future genetic gain in forest trees. Any gains that can accrue directly from genetic engineering, and be captured through propagation technology, will be superimposed on those from population improvement. Efficient population management will need to be directed not only at pursuing recognised breeding goals, but also in preparing for addressing new, unpredictable breeding goals. Both these goals are served by maintaining genetic diversity.

Genetic diversity depends initially on the population base. For characterising the base, genomics is a powerful adjunct, in conjunction with common-garden experiments. Within populations there are various possible measures of genetic diversity, involving polymorphisms for neutral markers and functional genes, and numbers of alleles and allele frequencies. Calibrating marker diversity against functional diversity is not simple, but will become easier with functional genomics in which the phenotypic impacts of nucleotide polymorphisms may eventually be identified. A need exists to counter the tendency for continued selection to erode diversity.

An associated, but not identical issue is controlling levels of inbreeding, which is not wanted in commercial material and yet may serve as a breeding tool. A customary approach is to pursue intensive ongoing genetic gain in a pedigreed breeding population underpinned by very broadly based gene resources. Use of genomics can relax some requirements for population management, notably the need for pair-crossing in order to maintain pedigree. Related to that, genomics can serve to retrieve historical losses of pedigree information, even overturning the tenet of production populations being genetic dead-ends. It can also correct potentially costly misidentification of material.

Genomic information has the widely hailed application of marker-assisted selection, although this has encountered various problems with forest trees. It is a promising tool in the quest not just for disease resistance but resistance that is durable. This requires a diversity of resistance mechanisms, yet the presence of some resistance genes can mask the presence or absence of others, unless the latter can be detected genomically.

With future knowledge of functional polymorphisms the new understanding of the nature and origins of genetic variation should allow far more efficient management of populations in order to both exploit genetic diversity and preserve it for the longer term.

Keywords: Population management, genetic diversity, genomics, DNA markers, pedigree

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## INTRODUCTION

Genomic information on forest trees can be used for several purposes, apart from being part of the advancing front of fundamental knowledge. Of main interest for this paper is how the availability of genomic research tools and the resulting information can serve the underlying objectives of population management which, in turn, is all about capturing cumulative genetic gain while maintaining the genetic diversity that safeguards the future genetic gains. For capturing genetic gain, genomic information can be used for marker-assisted or marker-based selection which exploits linkage disequilibria between assayable polymorphisms and ones that underpin phenotypic variability. Such selection can be refined into gene-assisted or gene-based selection if it has become possible to identify actual nucleotide polymorphisms that govern quantitative effects on traits. Much has been written about the issues of refining and quantifying the efficiency of selection based on use of genomic information, but for this paper that is of interest mainly for its ramifications for appropriate structuring of populations. Genomic information also has value for safeguarding the capture of genetic gains by verifying genetic identity. Of prime interest for this paper are the various more direct applications of genomic information for appropriately structuring populations.

The need for genetic diversity in forest tree breeding programs has never been seriously challenged, even if it has not always been honoured in how improvement programs are implemented. Long-term gain will depend on genetic diversity, which will tend to be run down by the process of intensive selection and associated maintenance of very finite breeding populations. Such limitations in long-term response to may not arise at all rapidly provided the same breeding goal continues to be pursued. However, breeding goals can change rapidly, owing to changes in market perceptions or biotic developments such as the emergence of significant fungal diseases or animal pests. Such changes can be substantial, and place heavy demands on available genetic diversity. While genetic engineering offers the prospect of conferring attributes that are not programmed by any of the available genetic diversity within species, the contributions of genetic engineering are widely seen as being properly superimposed upon a platform of genetic improvement resulting from classical breeding. For instance, engineering a new function from scratch can take a long time, and be a very uncertain venture compared with capturing the attribute from straightforward expression of existing genetic variation. On the other hand, genetic engineering technologies could also confer a narrow suite of attributes faster than existing breeding approaches can, although this may well impose added pressures of reduction in diversity, particularly in deployed populations.

Even without a specific or pressing problem, obtaining a meaningful measure of genetic diversity is highly desirable. Diversity can derive both from diverse population origins and within-population variation. Genomics can be used to indicate origins, of material of mixed ancestry. Traditional taxonomic tools, including chemotaxonomic ones, can be effective in this area, but genomic information has major advantages through often requiring limited tissue samples, providing quick results, and being independent of the multifarious environmental influences. Information on population origins needs to be complemented by information from common-garden field experiments which yield information on a range of traits that include growth potential, environmental tolerances, biotic resistances, and wood properties.

## MANAGEMENT ISSUES

The issues will be addressed under the following categories:

- Characterising the genetic base (sorting out taxonomy and crossability; identifying ancestral populations, migration history, and past adaptive pressures)
- Quantifying within-population diversity
- Managing inbreeding and relatedness
- Selection using markers
- Assuring durable disease resistance
- The future (in the era of functional genomics).

## GENETIC BASE

Knowledge of the genetic base that gave rise to one's population is always welcome, although not necessarily crucial or easily obtained. In the first instance, one may want to clear up the taxonomic status of one's material. Crossability, while an important but not absolute taxonomic criterion, is of immediate practical importance and something that needs to be addressed empirically. For addressing the general taxonomic issues, there are various classical herbarium-based criteria, which have come to be supplemented by phytochemical and cytological evidence, but genomic analysis is developing into a powerful new tool for the purpose. Even without a traditional taxonomic issue, it can be valuable, for instance, to know the geographic origins of a land-race population. For instance, the effects of sub-optimal origin may have been masked by either a shake-out of the neighbourhood inbreeding of natural populations, or by adaptive responses to selection pressures imposed by the exotic environment. Alternatively, a knowledge of the Quaternary migration history may warn one that a local population is likely to be significantly sub-optimal for commercial purposes. For addressing such issues, common-garden field experiments are likely to provide the best information, but genomic information may often provide a powerful adjunct, with the advantage of being far quicker to obtain.

Attempts to characterize different populations or even interfertile species for their collective diversity, without the benefit of common-garden field experiments, can be perilous. Apparent affinities among such entities can depend very strongly on the set of genomic markers chosen. Besides, the inherently neutral nature of many genomic markers, whatever their power for certain purposes, means that there is no *a priori* reason why they should indicate values for the adaptive or commercial traits that are of direct interest to the breeder. In a case that will not be named, a genomic study showed a particular tree species to be undistinctive with respect to the markers used. From that, the suggestion was made that the species could forgotten about for purposes of genetic conservation. This flew in the face of common forestry knowledge that the species concerned is highly distinctive in respect of certain ecological tolerances and silvical properties.

Indeed, indications are that neutral DNA markers can often be poor indicators of adaptive differences among populations within species (e.g. Karhu et al. 1996).

Where there is interest in preserving populations in a genetically pure state, genomics can serve as a new and powerful tool for detecting genetic contamination, quantifying it, and even culling individuals resulting from the contamination. This, however, will depend on identifying DNA markers that differentiate well between the populations in question.

## ISSUES OF QUANTIFTING WITHIN-POPULATION DIVERSITY AND IMPLICATIONS

Apart from population origins being a source of diversity that can be both identified and characterized, there is the within-population diversity, which may often be the bigger general issue. There remains the issue of whether marker diversity parallels the functional diversity that is of real interest to the breeder. But there are also issues of what are the most meaningful measures of diversity for markers and functional traits respectively.

Various possible measures of diversity exist, and they can have different implications according to the intensity of selection and the time frame of interest. For both markers and functional alleles there are numbers of alleles at the polymorphic loci, the expected heterozygosity over such loci which will often be dominated by intermediate allele frequencies, plus the numbers of loci involved. For functional alleles, the magnitudes of their effects are also of obvious importance.

Numbers of alleles per locus and expected heterozygosity are two, largely complementary measures. Numbers of alleles are readily influenced by sampling effects. As such, they are very subject to founder effects or genetic drift, which can both occur incidental to intensive selection. They will also be affected by sample sizes in population surveys.

Percentages of functioning genes that are polymorphic are still conjectural, at least for the typical outbreeding forest tree species. Nevertheless, this is an area where knowledge will doubtless make rapid advances (see later).

Potential response to selection depends on functional diversity. The immediate functional diversity, and consequent short-term responses to selection, will tend to be governed by alleles of intermediate frequency – unless there is both very intensive selection and alleles of large effect for the trait(s) under selection. Longer-term selection responses, however, may depend more on alleles that are currently at low frequencies but will only contribute significantly to expressed variation when their frequencies increase. The ways in which low-frequency alleles contribute to functional diversity will be especially sensitive to the degree to which the effects of such alleles are recessive or dominant.

In functional genes, many polymorphisms are silent, since they involve synonymous codons. Considerable discrimination is therefore possible between identity by function and identity by descent, which may offer important insights into the way in which different populations may have come to diverge.

Another issue is to what extent the functional genetic variation is governed by polymorphisms in the coding or promoter regions of genes. Some recent findings for plant species other than forest trees (e.g. Morgante & Salamini 2003, Paran & Zamir 2003) have indicated that large phenotypic variations can be governed by polymorphisms in the regulatory region rather than in the coding regions of the genes concerned. Such genetic variation will therefore not be evident in protein polymorphisms, although it could be detected via variation in transcript- protein- and/or metabolite concentrations in specific tissues.

## MANAGING INBREEDING AND RELATEDNESS

Apart from running down genetic diversity, long-term maintenance of closed, finite breeding populations inevitably incurs some level of inbreeding. Inbreeding is not necessarily the same thing as loss of diversity, because deliberate inbreeding can be practised to both maintain diversity and preserve options for subsequent outcrossing. Deleterious effects arising from inbreeding, namely loss in fitness and/or reproductive capacity via the effects of lethal or semi-lethal loci, could be managed using information from markers linked to such loci (e.g. Kuang et al. 1998). However, costs involved in establishing populations for detection of marker-trait associations are likely to be considerable relative to the gains from such selection, particularly in breeding populations. Nonetheless, as coancestry builds in such population, the imperative is likely to increase for more proactively dealing with the problem. Options range from avoiding certain allelic combinations to actively purging lethal genes.

For both managing inbreeding and maintaining genetic diversity, maintaining full pedigree information is extremely valuable. However, the traditional way of maintaining full pedigree has its costs, both direct and in achieving some of its aims. Making controlled pair-crosses, and keeping pair-crosses properly identified in replicated field experiments, are expensive operations, especially if the reproductive biology of a species makes pair-crossing difficult. In some cases, open pollination may even be preferred. Also, the size of fully pedigreed breeding populations may be constrained to levels at which loss of low-frequency alleles can become significant (Burdon 1997).

Even where full pedigree is nominally kept, misidentification can occur in various ways. That can compromise genetic gain, the more so the more advanced a breeding program is. It could also compromise control of inbreeding, and even maintenance of genetic diversity. However, simple-sequence repeat (SSR), or microsatellite, markers are proving a very powerful tool for verifying identity of both individual clones and parentage of progenies. Indeed, the misidentification rates being thus detected are quite embarrassing – something that is admitted much more freely by word of mouth than in print.

Genomic information, however, can allow retrieval of pedigree information. Lambeth *et al.* (2001) has advocated the use of DNA markers in order to allow retrieval of full pedigree information after saving costs by making polycrosses instead of pair-crosses. Earlier, Burdon (1997) had proposed use of DNA markers be used to make good a complete lack of pedigree information if it was necessary to select from unpedigreed commercial stands. This eventuality runs counter to a traditional tenet of commercial stands being a genetic dead-end in population

management. It could, however, arise in the case of a biotic crisis whereby only very rare trees were resistant to a new disease or pathogen strain. Such resistant individuals might be products of very rare favourable *de-novo* mutations, and finding them would likely depend on the sheer numbers of genotypes that would only be available in large commercial plantings. Pedigree reconstruction would allow the breeder to avoid selecting resistant trees all from one or a very few pedigrees with the attendant risks of both inbreeding and loss of genetic diversity. Having a finite set of possible or likely parents, as may be the case in seed orchards, would be a big advantage. That said, the challenges of achieving complete pedigree reconstruction may be formidable, but even partial success may suffice for achieving the underlying objective. Such a scenario may seem fanciful, but biotic crises have affected some very important tree species. For the breeder to tackle a biotic crisis head-on would also require powerful propagation technology and/or the lack of alternative species that are strongly competitive commercially.

Despite the obvious challenges in developing a sufficiently powerful set of markers for complete pedigree reconstruction, the sort of SSR marker diversity discovered in pines (Karhu et al. 2000) and and Douglas-fir (Slavov et al. 2004) augurs well.

## SELECTION USING GENETIC MARKERS

The potential benefits of using genomics to detect and use quantitative trait loci (QTL) for marker-assisted or marker-based selection (collectively referred to as MAS) are well recognised. It is widely agreed that there are tree general areas where MAS could be especially beneficial: increasing selection intensity, earlier selection, and cheaper selection for expensive-to-measure traits. However, various methodological traps have come to light (e.g. Ball 2001), and much had already been written about both the potential advantages and the pitfalls of MAS (e.g. Strauss et al. 1992, Johnson et al. 2000). The magnitudes of QTL effects are subject to estimation errors, and some can be greatly overestimated, in what is widely known as selection bias. However, experience with annual (or other short-term) agricultural crops has indicated that where QTL of large effects are involved, as may happen with introgressing genes from wild relatives into domesticated crops, some major payoffs can accrue (cf Paran & Zamir 2003).

Overall, MAS can be a selection tool for both breeding and production populations. While use of MAS as such is in no way central to population management, questions have been raised concerning the possibility that its intensive use would incur risks of otherwise avoidable loss of potentially useful alleles. On the other hand, as information on functional loci becomes increasingly available, tools to track specific alleles will become more and more effective, facilitating quantification and/or avoidance of allele loss during selection.

With forest trees, which are typically outbreeders, there is usually not the linkage disequilibrium between non-genic marker alleles and QTL that is required for MAS. However, with fusion of well-differentiated populations, or with advanced hybrids between species (as in domesticated apple - Bus et al. 2000) – there may well be cases of the desired combination of large-effect QTL and strong linkage disequilibrium. Another exception is within known pedigrees, for which linkages have to be identified in each individual case. This area has been the focus of much effort over the past decade, driven in part by the development of within-family marker-assisted

selection. Such research has also resulted in the development of marker tools that can reveal much about population structure and existing inbreeding levels. However, these marker systems are largely based on polymorphisms in non-gene-associated regions, and may not adequately reflect the underlying functional variation.

More recently, research has changed focus from pedigree-specific populations, to non-pedigreed association genetics. One of the reasons for this is the desire to develop the option of among-family selection. The marker systems concerned would be more likely to be based on expressed genes that have a suspected role in trait variation. The benefit of assaying polymorphisms within and associated with expressed genes, together with non-structured populations for ascertaining marker-trait associations, is that key parameters such as gene and nucleotide diversity, heterozygosity, inbreeding and coancestry relationships can be calculated (Neale & Savolinen 2004). Therefore, the experimentation required for gene-assisted among- and within-family selection has the added benefit of revealing key information regarding those experimental populations.

A key constraint to the implementation of markers as selection tools, is the substantive up-front costs required to detect marker-trait associations (Johnson et al. 2000, Wilcox et al. 2001). As such the more likely implementation is in deployed populations as opposed to breeding population advancement, because of earlier commercial returns. If marker-trait associations are used for selection in the breeding population, a build-up of coancestry may be hastened because of unequal selection rates in specific pedigrees. Such a build-up would need to be judiciously countered in order to maintain diversity.

## DISEASE RESISTANCE (DURABILITY)

A related issue is ensuring durability of disease resistance against genetic shifts in the pathogens (e.g. Burdon 2001). While there is no certainty that lack of durability will be a problem in any particular case, measures to ensure durability seem prudent. Durability typically appears to depend strongly on a diversity of resistance mechanisms. For large-scale deployment of individual clones, pyramiding a multiplicity of resistance factors within individual clones is a likely need. For heterogeneous populations, it appears that population resistance is not dependent on a multiplicity of resistance factors all being present in every individual. Either way, however, knowledge of the various resistance factors, how they operate, and how to detect their presence, is extremely valuable. It is one of the problems with disease resistance that some of the more spectacular forms of resistance are vulnerable to pathotype shifts and yet tend to mask the presence of other resistance factors that may be needed in order to ensure durability. For addressing this general problem, genomic information when combined with infection studies in genetic experiments can be extremely powerful, and its use is progressing rapidly with annual crops. This is partly because there are often individual resistance genes of substantial effects in situations where there are superficial appearances of polygenic resistance. With the loblolly pine/fusiform rust pathosystem progress is being made in identifying genetic factors involved in resistance (Wilcox et al. 1997, Schmidt 2003). On the one hand, the short period of the pine host susceptibility during the rotation may make for a relatively simple pathosystem, and one in which the pine is not unduly vulnerable to rapid pathotype shifts (Schmidt 2003); on the other

hand, acquiring good data is relatively slow and difficult. In the case of leaf rusts affecting poplars more rapid research progress is apparently being made (e.g. Yin et al. 2004). Apart from poplars having come to be treated widely as model species, the biology of the rusts is conducive to much more rapid acquisition of data.

With the search for resistance to blister rust on North American white pines much progress has been made by classical genetic studies. Various individual resistance genes have been identified, and the nature of their action (e.g. dominance or recessiveness) characterised (Sniezko et al. 2004). This, however, has been a long, drawn-out process. It is to be hoped that, with genomic tools available, future progress can be much more rapid, in both research and its applications.

For deployment of genetically resistant material the diversity of resistance mechanisms can, in principle, be used judiciously. Comprehensive pyramiding of resistance genes in individual genotypes cannot be expected to come quickly. But, even without such pyramiding, it should be possible to deploy mixes of tree genotypes that can be expected to show good population resistance.

## THE FUTURE

Current population management is based largely upon various suppositions, which are often tacit rather than explicit, concerning the nature of genetic diversity and the ways in which it can be generated. The usual rule of thumb is for production populations to contain a (very) few tens of effectively unrelated individuals, breeding populations a few hundred, and gene resources a few thousands (e.g. White 1992). It tends to be assumed that mutation is a rare event at any individual locus, but that low-frequency alleles are potentially very important. Yet there are various long-term selection experiments, in which continued response has occurred for many generations in closed populations, which suggests that significant mutational or quasi-mutational events may not be rare. Since maintaining breeding populations and gene resources is expensive, and likely to become more so as progressive genetic gain increases the opportunity costs of maintaining such material, the question of requisite populations sizes and appropriate management systems is far from trivial.

Questions of both population management and capture of genetic gain for operational use will be increasingly addressed by extending genomics into the study of functional genes. There are various ways in which this can be done. First, the genes need to be identified and located, and then their polymorphisms can be studied. Starting with just with information on tight linkages still leaves very long sequences to investigate and interpret. However, with libraries of expressed sequence tags (ESTs), belonging to genes whose function has been identified in model species, there are now much more powerful tools for locating specific genes. The quintessential model plant species has become Arabidopsis which, despite not being closely related to conifers, shows remarkably high levels of orthology with the full range of forest trees (e.g. Kirst et al. 2003), even though much of the developmental physiology and morphology appear very different. However, with the availability of the full nuclear and organelle genomic sequences of poplar (*Populus trichocarpa*) (http://www.jgi.doe.gov/poplar), it is likely that this will become the model species of choice for certain purposes. Among conifers, there are not only the

orthologies but also some remarkably high levels of synteny (e.g. between loblolly and Monterey pines). Thus, genes of common functions tend to be located in essentially the same positions on the same chromosomes of the respective species, so genomic information obtained from one species can be easily used for studying another. With the identity and roles of various genes identified, the developmental significance of polymorphisms can then be ascertained. Tactically, that can allow the breeder to screen for particular alleles, or to identify alleles that might be transferred between species by genetic transformation.

The value of orthologies for probing the nature of polymorphisms has its limits, however, since the roles of particular genes in governing trait phenotypes can vary among plant species. Experimentally, the roles of different base-sequence alleles in governing phenotype can be probed or verified by using genetic transformation; however, some of the information may take a long time to come in with trees, particularly for traits that are expressed only at later ages and where no early indicator characteristics can be used.

Various strategic implications will stem from the basic information on the levels and nature of polymorphism, on the dynamics and nature of mutation and on gene transposition. Knowing the level and nature of polymorphisms involving gene function, as distinct from recent descent, should give better insights into the significance of numbers, at different stages in the life cycle, in population management.

We have various implicitly or explicitly pedigree-based measures of effective population size, which serve as indicators of changes in genetic diversity. These measures include Status Number (Lindgren et al. 1996), as well as more traditional measures (Caballero 1994). Although we have some intuitive appreciation of the value of different measures for different purposes, their exact practical significance is usually very uncertain. Hopefully, with knowledge of the level and nature of functional polymorphisms, and of mutational processes and transpositions, it will be possible to achieve not only better and more direct measures of genetic diversity but also much better interpretation of the measures of effective population size.

## CONCLUDING

For many of those who are charged with population management, or see it as their responsibility, a strongly precautionary approach is indicated in the present state of genomic knowledge. At the same time, breeders may have to deal with people who want to see guarantees of returns from the direct costs and opportunity costs involved in population management, which can place them in a difficult situation. Better knowledge will therefore be extremely welcome. As yet we have no silver bullet, and we may never be able to majorly relax the requirements for sizes of the populations needed for continuing advance of breeding programmes. Nonetheless, the very rapid progress that is now occurring in functional genomic promises a much better basis for future decision-making.

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# SSR analysis of pollen parent fitness in polymix crosses of *Eucalyptus grandis* and *E. urophylla*

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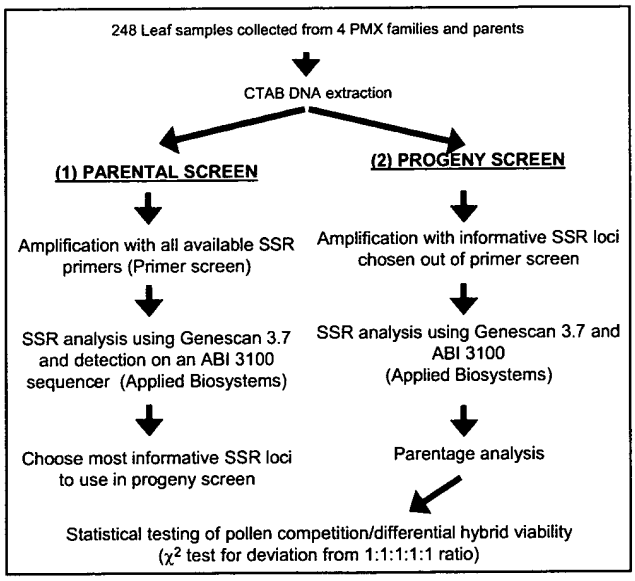
## Summary

Polymix (PMX) breeding of interspecific hybrids of forest trees promises to greatly reduce the cost of controlled crosses, while increasing the number of hybrid genotypes that can be evaluated. However, severe pollen competition may make this approach impractical, as dominant pollen parents may not necessarily produce superior hybrid offspring. In this study, we used simple sequence repeat (SSR) marker fingerprinting and parentage analysis to determine the relative contribution of pollen parents in three 5-parent mixes (A, B and C) of *E. grandis* pollen used in four interspecific crosses with three *E. urophylla* seed parents. Sixteen SSR marker loci were tested, of which four were found to be informative enough to uniquely assign 247 of 248 tested $F_1$ polymix progeny to their respective seed and pollen parents. We found that the contribution of pollen parents to each polymix progeny set was highly biased in favour of one or two dominant pollen parents, while several pollen parents did not produce any hybrid progeny. The relative fitness of the pollen parents was remarkably constant in two crosses where the same *E. grandis* pollen mix was used to pollinate two different *E. urophylla* seed parents. These results suggest that severe pollen competition and/or differential hybrid viability occur in polymix crosses of *E. grandis* and *E. urophylla* and that this phenomenon may be independent of the maternal genotype. It may also be necessary to re-evaluate polymix approaches to hybrid breeding in *Eucalyptus* and to investigate the possibility of using balanced pollen mixes of parents with equivalent fitness in interspecific crosses.

## Materials and Methods




### PMX Family Structure

| PMX Family | Cross[a] | Individuals |
|---|---|---|
| 1 | UP-272 x PMX A | 74 |
| 2 | UP-273 x PMX C | 55 |
| 3 | UP-274 x PMX B | 37 |
| 4 | UP-274 x PMX C | 82 |
| | TOTAL | 248 |

[a] UP-272,273 and 274 are three *E. urophylla* seed parents used in crosses with the three 5-parent pollen mixes (PMX A, B and C) of *E. grandis*. Note that PMX C was used in two different crosses

### Power of Detection

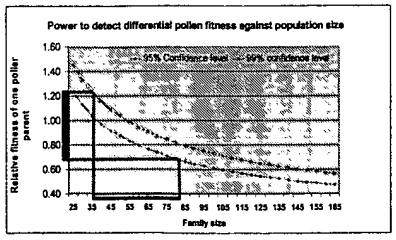



**Figure 1.** Lower limits of detection of relative difference in pollen parent fitness (vertical green bar) determined by the range of family sizes tested in this study (horizontal green bar)

## Results and Discussion

- Family sizes ranged from 37 to 82, which set a lower limit of detection of differential pollen parent fitness to a range of 1.2 to 0.7 (depending on family size) – (Figure 1)
- The four SSR loci selected were highly informative with individual allele frequencies mostly <0.15 and, in combination, allowed unique assignment of progeny to seed and pollen parents – (Figure 2)
- We observed a significant deviation from the expected 1:1:1:1:1 pollen parent ratio for all 4 families – (Figure 3)
- Some parents made no pollen contribution. This could be due to low pollen parent fitness (pollen competition or low viability of hybrid progeny produced by these pollen parents)
- We observed significant allelic transmission ratio distortion at two of the SSR loci (data not shown). This could be caused by selection on individual alleles due to linkage to pollen fertility or hybrid viability factors.

### Parentage Analysis Using SSR Marker Analysis

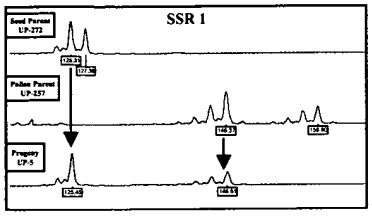



**Figure 2.** Example of electropherograms obtained after SSR analysis of parents and polymix progeny on the ABI 3100 automated sequencer and Genescan software. The inheritance of parental alleles is indicated with vertical arrows.

### Relative Fitness of Pollen Parents

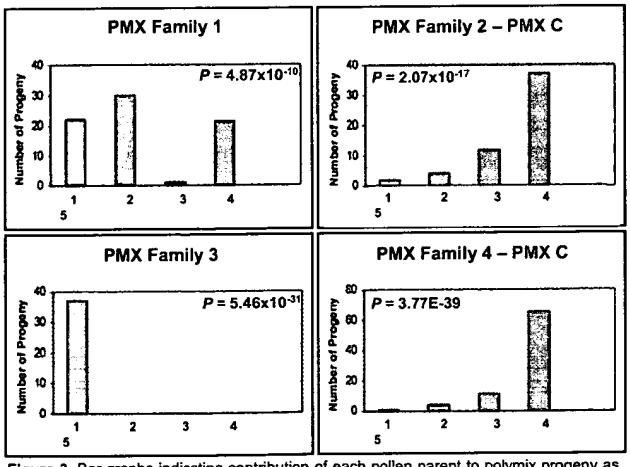



**Figure 3.** Bar graphs indicating contribution of each pollen parent to polymix progeny as determined using SSR marker analysis. A significant tendency towards a dominant parent is visible. In family 2 & 4 the same PMX was used and similar results were observed, suggesting that the differential pollen fitness is independent of the maternal genotype. The *P*-values of the $\chi^2$ test at the 0.05 level of significance are indicated.

## Conclusions

- **Four informative SSR loci were sufficient to uniquely assign 247 of 248 polymix hybrid progeny to 3 seed parent and 15 pollen parents**
- **Large differences were observed in the relative fitness of pollen parents in pollen mixes as measured by the relative contribution of each pollen parent to polymix progeny**
- **This could be problematic for PMX breeding since some genotypic combinations may be severely underrepresented**
- **A possible solution would be to make "balanced" pollen mixes with parents of similar pollen fitness**

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